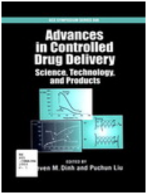

Advances in Controlled Drug Delivery

Science, Technology, and Products

EDITED BY

even M. Dinh and Puchun Liu

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Advances in Controlled Drug Delivery

Science, Technology, and Products

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American Chemical Society, Washington, DC

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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Preface

Controlled drug delivery is intended to maximize the therapeutic effects of a drug molecule while minimizing its side-effects by addressing three fundamental parameters: concentration, duration and site of action (*1*). In addition to developing technological solutions, simplicity in the final product needs to be maintained in order to ensure patient compliance and to achieve the desired goals of treatment.

The drug delivery market is going through a rapid transformation from a trivial branch to a necessary part of the pharmaceutical industry. The market is expected to rise exponentially from $19 billion in 2000 to reach nearly $42 billion in 2007 (*2*). The unveiling of the human genome and discovery of gene therapy for diseases is expected to further advance the development of drug delivery technologies to create novel pharmaceutical products.

This book is the result of a symposium entitled *Advances in Controlled Drug Delivery* held during the American Chemical Society (ACS) National Meeting in San Diego, California in April 2001. This book spans work in many diverse areas. Each of the ten chapters is written by one or more industrial and academic scientists who have developed and contributed to the advances in controlled drug delivery. Important topics are covered such as gene delivery, alternative routes for macromolecules, tissue engineering and cell targeting, and novel chemistry.

Chapter 1 covers the work of delivering heterologous tissue inhibitor of metalloproteinase (TIMP). The naked NDA-mediated gene therapy is promising as it is non-toxic and has fewer side effects than virus-mediated gene therapy. Targeted gene therapy for systemic delivery of proteins by intramuscular injection of expression plasmids provides a convenient method of studying the therapeutic functions of specific proteins.

Chapter 2 covers the use of gene microarrays for assessing heparin's ability to inhibit intimal hyperlasia following arterial injury. This tool allows the screening of thousands of mRNA expression levels in a

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single sample. As the ability to analyze the vast amount of data generated increases, so will our ability to design drug formulation and delivery methods to target disease.

Chapter 3 demonstrates that oral heparin is an effective agent against neointimal hyperplasia following balloon induced injury and the more chronic deep injury associated with stent implantation in a rabbit iliac artery model. Its pharmacokinetic and pharmacodynamic profiles are more favorable than that of subcutaneous or intravenous injection.

Chapter 4 shows a non-transportable drug, which is chemically linked to a transportable peptide-vector for delivery across the blood–brain barrier. As potent macromolecules cannot be effectively delivered to the brain by conventional means, continuing refinement of new delivery methods will be essential to realizing the potential of these macromolecular drugs.

Chapter 5 presents the pulmonary system that can be tailored to deliver macromolecules or small molecules either systemically or topically at high-delivery efficiency. Development of improved aerosol delivery systems will increase efficiency and reduce variability as well as target the drug to a specific region of the lung to reduce side effects.

Chapter 6 describes potential growth factors and polymers that can be adopted in tissue engineering approaches. Dynamically controlled growth factor delivery is the future perspective of growth factor delivery in tissue engineering as well as in cell transplantation. Development of polymer scaffolds that release growth factors in response to mechanical stimulation provides a novel means to guide tissue formation in vivo.

Chapter 7 presents a new modality of drug targeting to tumors. This method is based on drug encapsulation in polymeric micelles followed by localized release at the tumor site triggered by ultrasound. Suppression of the intracellular energy metabolism and the permeabilization of the cytoplasmic acidic vesicles enhance drug uptake and trafficking into cell nuclei.

Chapter 8 covers a novel chemistry of preparing delivery agents for oral delivery of macromolecular and polar drugs. This method eliminates the preparation of acid chlorides, and is amenable for large-scale preparation providing excellent yields of the desired carboxamides.

Chapter 9 describes the application of this technology in topical drug delivery. As a remarkable example of polymers, silicones have proven their suitability in the preparation of various types of drug delivery

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systems, from simple creams to more complex transdermal patch or subcutaneous implanted devices.

Chapter 10 covers a versatile and fully degradable system obtained from water-soluble dextran grafted with oligolactic acid chains. With tailored mechanical and degradation properties, hydrogels that are physically cross-linked by stereocomplex interactions have attracted recent attention for drug delivery purposes.

The future of drug delivery technologies will in essence be directed at solving problems of long extent in the field, namely drug targeting to a specific organ or tissue (spatial) and better controlling of the rate of release (temporal). The future is bright, but more challenging. Advances in these technologies will directly impact peptide–protein delivery as well as making gene therapy a reality. With this book, we hope that bringing together experts in these diverse fields, and initiating direct new dialogues among them will contribute to further advancement of controlled drug delivery technologies.

We thank the authors for their participation at the symposium and for their contributions to the chapters of this book.

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Chapter 1

Naked DNA-Mediated Gene Therapy: Clinical Application of Tissue Inhibitors of Matrix Metalloproteinase

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After all, once the first phase of Human Genome Project (sequence) are finished, the obvious questions become what are the functional relevances of these proteins and how to deliver these functional proteins. While the functional relevance of the protein is a very important aspect of the Proteomics, developing of a new delivery platform for administration of functional proteins faces even bigger challenge. Recombinant protein held out the promise of therapeutics based on biology rather than chemistry. However, the cost-effective manufacturing of active proteins at large scale has been a technical hurdle and the short half-life of many proteins in the blood has been a bigger biological hurdle to the widespread development of therapeutic proteins. Gene therapy aimed on gene delivery into living tissues has significant implications in various clinical applications. Although the current virus-mediated gene delivery is the major route for gene therapy, it faces a limitation due to the serious side effects. Among the nonviral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA has been widely explored. Tissue inhibitor of metalloproteinase (TIMP) is a secreted protein, which specifically inhibit matrix metalloproteinase (MMP). Overexpression of MMP has been linked to many diseases such as cancer, cardiovascular restenosis, arthritis, and inflammatory diseases. Systemic delivery TIMP gene by a single intramuscular injection of TIMP DNA will be discussed in the animal models of cancer and arthritis.

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Naked DNA-mediated gene therapy

Gene delivery into living tissues has significant implications in gene therapy and various clinical applications. Among the nonviral techniques for gene transfer *in vivo*, the direct injection of plasmid DNA into muscle provides a simple, inexpensive, and safe method (*1*). The postmitotic nature and longevity of myofibers permits the stable expression of transfected genes, although the transfected DNA does not usually undergo chromosomal integration. Delivery of heterologous genes into skeletal muscles has been shown to provide sustained systemic administration of proteins in the circulation. Intramuscular injection of an interleukin-5 (IL-5) expression plasmid results in IL-5 production in muscle at a level sufficient to induce marked proliferation of eosinophils in the bone marrow and eosinophil infiltration of various organs (*2*). A single intramuscular injection of an erythropoietin expression plasmid into adult mice produced physiologic elevations in serum erythropoietin levels and increased hematocrits (*3*). Most recently, administration of the secreted form of the angiogenesis inhibitor endostatin results in sustained expression and secretion of endostatin into the blood circulation and a systemic inhibition of tumor growth and metastases (*4*). These results suggest that intramuscular DNA injection is a useful method of delivering cytokines, growth regulators, and other serum proteins systemically.

One of the major obstacles of intramuscular gene transfer is the relatively low expression level of the transfected gene. Conditions that affect the efficiency of gene transfer by intramuscular DNA injection have been analyzed. Among several favorable factors including injection of expression plasmids into regenerating muscle and formulation of expression plasmids with synthetic polymer, electroporation has generated some excitement of improving the gene transfer efficiency of intramuscular DNA injection (*1*). Electroporation has been widely used to introduce DNA into various types of cells *in vitro*. Gene transfer by electroporation *in vivo* has been effective for introducing DNA into mouse skin (*5*), chick embryos (*6*), rat liver (*7*), and murine melanoma (*8*). Aihara H et al has recently examined the efficiency of IL-5 gene transfer into muscle by electroporation *in vivo*. Electroporation after plasmid injection increased serum levels of IL-5 more than 10-fold compared with that of simple intramuscular plasmid injection (*1*).

Tissue inhibitors of matrix metalloproteinase (TIMP)

Matrix metalloproteinases (MMPs) play a critical role in extracellular matrix (ECM) homeostasis. Controlled remodeling of the ECM is an essential aspect in process during normal development, and deregulated remodeling has been indicated to have a role in etiology of diseases such as arthritis, periodontal disease, and cancer metastasis (*9-13*). The down-regulation of MMP may occur at the level of transcriptional regulation of the genes; activation of secreted

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proenzymes; and through specific inhibitor proteins, such as TIMPs. TIMPs are secreted multifunctional proteins that play pivotal roles in the regulation of ECM metabolism. Their most widely recognized action is as inhibitors of matrix MMPs. Thus, the net MMP activity in the ECM is controlled by the balance between activated enzyme levels and TIMPs levels. Four mammalian TIMPs have been identified so far: TIMP-1 (*14*), TIMP-2 (*15-16*), TIMP-3 (*17-19*), and the recently cloned TIMP-4 (*20-22*). The proteins are classified based on structural similarity to each other, as well as their ability to inhibit metalloproteinases. Like MMPs, the expression of TIMPs in the tissue is also controlled during tissue remodeling and physiological conditions to maintain a balance in the metabolism of the extracellular matrix (*23*).

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Suppression of tumorigenesis by TIMP

The overproduction and unrestrained activity of MMPs have been linked to malignant conversion of tumor cells (*24-29*). Decreased production of TIMP could also result in greater effective enzyme activity and invasive potentials (*30-31*). These results suggest that the inhibitory activity of TIMPs might be important in inhibiting tumor malignant progression leading to invasion and metastasis. In fact, tumor invasion and metastasis can be inhibited by up-regulation of TIMP expression in tumor cells (*22,32-33*). Intraperitoneal injection of TIMP-1 and TIMP-2 recombinant protein has been shown to inhibit lung colonization of B16 melanoma cells (*34-35*). Overexpression of TIMP-1 inhibits tumor growth and metastasis of melanoma (*36*), suppresses metastatic ability of human gastric cancer cells (*37*) and oral squamous cell carcinoma (*38*). Adenoviral transfer of TIMP-3 into Hela, fibrosarcoma cell line HT1080, and melanoma cells inhibits the invasive ability of those cells and induces apoptosis (*39-40*). Overexpression of TIMP-4 in a human breast cancer cell line MDA-MB-435 cells by cDNA transfection inhibits the invasion, metastasis, and tumor growth (*22*).

In addition to inhibiting tumor cell invasion and metastasis, overexpression of TIMPs also inhibits primary tumor growth (*22,36,40*). Both invasiveness and tumorigenic potential of murine 3T3 cells are conferred when TIMP production is impaired by an antisense approach (*30-31*). *In vivo* tumorigenic and metastatic potentials of human breast cancer cells can also be inhibited by overexpression of a single TIMP-4 gene (*22*). There are several mechanisms that may attribute to the anti-tumor effect of TIMPs. First, there is evidence supporting a role for MMPs in early stages of tumor onset and primary tumor growth. Specifically, MMP-3 and MMP-9 have been reported to be involved in mammary carcinogenesis (*28*), skin carcinogenesis (*29*), and carcinogenesis of pancreatic islet (*41*). MMP-3 promotes spontaneous premalignant changes and malignant conversion in mammary glands (*28*). MMP-9, which is upregulated during early neoplastic progression in skin, stimulates tumor growth of oncogene-expressing keratinocytes (*29*). Second, MMPs also play a critical role in tumor-induced angiogenesis (*41*), and therefore TIMP-induced tumor inhibition may be mediated by inhibition of angiogenesis.

Intramuscular TIMP-4 gene therapy provides high and sustained circulating TIMP-4 protein levels

Although the inhibitory effects of TIMPs on tumor growth and metastasis are achieved by transfection of the respective TIMP gene into tumor cells, potential therapeutic applications of TIMPs for cancer treatment are limited by the lack of a method for systemic administration of TIMPs that can reach distant tumor locations. For systemic administration of TIMP-4, we undertook a gene therapy approach through intramuscular administration of TIMP-4 expression plasmid (*42-43*). In this regard, 50 microliters (150 μg) of TIMP-4 plasmid DNA (TIMP-4 cDNA in pCI-neo mammalian expression vector, Promega Corporation, Madison, WI) or empty vector (the parental plasmid) as a control were injected into the bilateral tibialis anterior muscles of 6-week old of female nude mice using a disposable insulin syringe with a 25-gauge needle. For electroporation, a pair of electrode needles was inserted into the muscle with a 5 mm gap within the DNA injection sites, and electric pulses were delivered using an electric pulse generator Electro Square Porator ECM 830 (Genetronics, Inc. San Diego, CA). Three pulses of 200 V each were delivered to the injection site at a rate of one pulse per second, each pulse lasting for 50 ms. Then, three pulses of the opposite polarity were applied. Plasma was collected prior to the injection, and at 7 and 14 days following the injection. TIMP-4 protein levels were determined by Western blot analysis. We have demonstrated that while there was no detectable TIMP-4 protein in the plasma prior to the injections, a significant amount of TIMP-4 was detected in the plasma at 7 days following the injection and continued to be present at 14 days post injection (*42-43*). Our data indicated muscle cells transfected with the TIMP-4 expression plasmid could produce and maintain high TIMP-4 protein levels in the circulation for at least 2 weeks after a single injection.

Tumor suppressing effect of intramuscularly administered TIMP-4 gene.

The potential anti-cancer therapeutic effects of electroporation-mediated TIMP-4 plasmid injection needed to be evaluated in experimental tumors. Since TIMP-4 inhibited the growth of G401 Wilms' tumor cells *in vitro* (*42*), we were interested in whether the similar tumor suppressing effect could be achieved *in vivo* by using the gene therapy approach through intramuscular administration of TIMP-4 expression plasmid. We took a preventive approach where TIMP-4 plasmid was injected prior to the tumor development. Three days following the injection of either TIMP-4 plasmid or control plasmid into nude mice, we injected G401 cells subcutaneously. We repeated the plasmid injections every 10 days until the animals are sacrificed. After a lag phase of 6-9 days, all animals in both groups (control plasmid or TIMP-4 plasmid injected) developed tumors in all injection sites (100%, i.e. 20 of 20 injections). After a slow growth phase of 14 days, tumors in control mice increased in volume at an exponential rate. In contrast, the growth rate of tumors in TIMP-4 injected mice

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was significantly slower. Seven weeks following tumor cell injection the size of tumors in TIMP-4 injected mice was only 53% of that in control mice (*42*). At week 7, animals were sacrificed and tumor tissue was analyzed for TIMP-4 immunostaining. Consistent with tumor data, strong immunohistochemical staining of TIMP-4 was observed in xenografts from TIMP-4 injected mice, while no TIMP-4 signal was detectable in xenografts from control mice.

We have shown that electroporation-mediated TIMP-4 gene delivery can produce sustained levels of circulating TIMP-4 protein. Furthermore, a significant amount of TIMP-4 protein was accumulated in tumor xenografts. The level of expression was sufficient to inhibit the growth of Wilms' tumor. The serum TIMP-4 levels obtained following the plasmid injection was significantly higher than the amount used in the *in vitro* experiments. However, TIMP-4-mediated *in vivo* tumor inhibition (about 50%) was less potent than its *in vitro* cell growth inhibition (about 90%). This discrepancy may be due to a difference in amount of TIMP-4 protein available to the target tumor cells. In the *in vitro* assay, the exact amount of TIMP-4 protein delivered to the tumor cells is controlled by the amount of purified protein added to the culture media. In the intramuscular gene therapy approach, to achieve effective tumor suppression, TIMP-4 gene has to be first expressed in skeletal muscle cells, secreted into blood circulation, reach the tumor environment, and penetrate into the solid tumor. As TIMP-4 leaves the circulation, it needs to cross a vast amount of extracellular matrix that contains MMPs before reaching the target tumor cells. The fate of TIMP-4 within the extracellular matrix is currently unknown, and it may be sequestered by the MMPs or be degraded to some degree before reaching the tumor cells. Therefore, the amount of circulating TIMP-4 may not indicate the actual amount of TIMP-4 available to the tumor cells. In addition, the tumor suppressing effect of TIMP-4 may also involve additional events other than the direct growth inhibition. The *in vivo* tumor growth is open to a variety of pro- and anti-tumor events such as angiogenesis, which may be inhibited by the high serum TIMP-4 levels (*22*).

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Complex roles of TIMPs in Cancer

TIMPs inhibit the activity of MMPs, which leads one to expect that an increase in the amount of TIMPs relative to MMPs could function to block cancer progression and therefore a potential TIMP-mediated cancer gene therapy. Although there are many data that indicate an increase in the amount of TIMPs could function to block cancer progression, there is also experimental evidence suggesting that TIMPs may function in a manner that promotes rather than suppresses tumor growth. The effects of TIMPs on tumorigenesis are multifunctional and paradoxical. Contrasting with their anti-MMP and anti-tumor activity, TIMP may also function in favor of tumor growth at three aspects, including (a) anti-apoptotic effect; (b) inhibition of angiogenic inhibitor (such as angiostatin and endostatin) converting MMPs and therefore stimulation of angiogenesis; and (c) involvement of activation of pro-MMP-2.

Unexpectedly, we recently demonstrated that systemic delivery of TIMP-4 by intramuscular administration of naked TIMP-4 DNA significantly stimulated mammary tumorigenesis *in vivo* (*43*). Consistent with its tumor stimulatory effect, TIMP-4 upregulates Bcl-2 and Bcl-X_L protein and protects breast cancer cells from apoptosis both *in vitro* and in nude mice.

The paradoxical effect of TIMPs on tumorigenesis can be reviewed from two *scenarios*. The first scenario correlates with the bioavailability of TIMP protein in tumor microenvironment. TIMPs have both anti-MMP activity, which favors the tumor-suppressing effect, and anti-apoptosis activity in some tumor cells such as human breast cancer cells (*43*), which exerts pro-tumor effect. The balance between anti-MMP and anti-apoptotic effect on tumor growth may depend on the amounts of bio-available TIMP protein in tumor microenvironment. In this regard, higher levels of TIMPs may have tumor suppressing effect, due to its dominant anti-MMP activity, while lower levels of TIMP may favor tumor growth, due to its anti-apoptotic activity. When a gene transfection approach is taken, it usually results in a selection of the most highly TIMP-expressed clones. The overexpression of TIMPs locally in every cancer cells would generate abundant inhibitory proteins in the tumor-stromal interface where the pro-tumor MMP activity is blocked. In contrast, in the intramuscular gene therapy approach, TIMP protein has to cross a vast amount of extracellular matrix proteins and circulation before reaching the target tumor cells, in which the anti-MMP function of circulating TIMP may be neutralized in part by circulating MMPs. Therefore, the amount of TIMP bioavailable to the tumor cells may be much lower than that from locally expressed TIMP in transfected cells and the balance was shifted in favor of its anti-apoptotic activity on tested breast cancer cells when it reached the tumor (*43*). However, in the case of G401 Wilms' tumor (*42*), systemically administrated TIMP-4 gene can inhibit tumor growth due to the lack of anti-apoptotic activity of TIMP-4 on G401 cells.

The second scenario correlates with the timing of TIMP function. In tumor xenograft model, when tumor cells are inoculated into animal or spread to a secondary organ, many tumor cells will suffer from apoptosis, due to the host reaction. Therefore, the anti-apoptosis effect may be more important during the tumor onset and early tumor growth. Once a tumor is established either in a secondary organ or in animal challenged with tumor cells, the antiapoptosis activity of TIMP is supposed to have little influence on tumor progression at this stage. On the contrary, sustained angiogenesis is far more important for tumor growth at later stage. Therefore, the antiangiogenesis activity of TIMP may become a dominant negative factor inhibiting tumor growth at this stage. Consistent with the timely differential dual functions, it has been demonstrated that TIMP-1 overexpression was of biphasic functions on lymphoma tumorigenesis. TIMP-1 has a significant tumor-stimulating effect during the tumor onset, but suppresses the tumor growth during the late state of tumor progression (*44*). These data support the timely divergent functions of TIMP on tumorigenesis.

Although there are many data which indicate an increase in the amount of TIMPs or MMPIs could function to block cancer progression, there is also

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experimental evidence suggesting that TIMPs may function in a manner that promotes rather than suppresses tumor growth. Whether the tumor stimulatory functions can prevail in the tumor environment and surpass the tumor-suppressing functions will direct the net functions of TIMP toward tumor promotion or tumor suppression. There are several possible factors which may affect the net function of TIMPs, including local concentration of TIMPs, cellular and pericellular distribution, the forms of presence in tumor and in circulation, the presence of a possible putative TIMP receptor on tumor cells, and the time when TIMP is available during the tumor onset and progression. Given these paradoxical facts, systemic administration of TIMP clinically may inhibit the invasion of tumor cells and the growth of established tumor, on condition that the tumor-suppressing effects are dominant. Otherwise, it may not inhibit tumor growth, even enhance tumor cells survival and therefore promote tumorigenesis in some patients. Future direction should focus on a new conceptual basis for design of strategies that use TIMP as a probe for MMP function in cancer gene therapy. Such directions include the dissection of the TIMP structure domains for its tumor-suppressing anti-MMP effect and the anti-apoptotic and thus pro-tumor activity. In this regard, potential cancer treatment by intramuscular delivery of truncated or mutated TIMP genes that are lacking the pro-tumor anti-apoptotic effect warrants further investigation.

TIMP-4 gene therapy prevents development of adjuvant-induced arthritis

The mainstay of arthritis development is destruction of joint surface followed by ineffective repair processes. This tissue remodeling when gone unchecked may cause further deterioration of the joint surface leading to complete loss of joint space making it a major target for therapeutic intervention. MMPs are the predominant group of enzymes that play a key role in both the destruction of the joint surface and the repair processes in rheumatoid arthritis pathogenesis. The process starts with activation of macrophages that secrete cytokines such as TNF-α and IL-1α, which in turn stimulate the expression of MMPs (*45-46*). Attempts are underway to inhibit the expression of proinflammatory cytokines (*47-49*) or the activity of MMPs (*50-53*) to stop the deterioration at its early stages. Studies have shown a causal role for MMPs in the pathogenesis of rheumatoid arthritis as determined by the findings that: 1) Synovial fluid obtained from patients with rheumatoid arthritis contains higher amounts of MMPs as well as TIMPs but the net ratio of MMP/TIMP is significantly increased (*54-57*); and 2) The circulating levels of certain MMPs and TIMPs are also increased in patients with rheumatoid arthritis as compared to non-immune arthritic patients or normal controls (*58-61*).

An imbalance between the MMPs and TIMPs in favor of the MMPs appear to be the major force in extracellular matrix destruction of arthritis. TIMPs can block the activity of MMPs, the inhibitory activity of TIMPs might be important in inhibiting arthritis. This rationale promoted us to investigate the potential therapeutic role of systemic administration of TIMP-4 gene on suppression of arthritis. We tested the effect of intramuscularly injected TIMP-4 gene on adjuvant-induced arthritis in rat model (*62*). Before the adjuvant treatment, rats were either injected with plasmid alone as a control group or injected with the plasmid containing the TIMP-4 gene as the treated group. In two consecutive experiments, all of the control animals (8/8) developed swelling of paws with an average diameter of 9.5 mm 10-14 days following the inoculation of the adjuvant. None of the animals in the TIMP-4 gene therapy treated group developed arthritis (0/10) as indicated by normal paw diameter sizes of average diameter of 5.5 mm. Clinical observations were further confirmed by histological evaluation of joint tissue in these animals. Normal rats and rats with adjuvant plus TIMP-4 injection showed an intact cartilage surface with no new bone formation or resorption surface, and no inflammatory cellular infiltration. Control animals treated with adjuvant plus control plasmid vector showed significant inflammatory reaction as evidenced by infiltration of the distal tibia, cuboid bones with inflammatory cells; infiltration of joint surface with macrophages; and severe erosion of cartilage in this area.

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Summary

Although transfection of expression vectors to cells provides effective means of studying disease pathogenesis, it remains impractical in therapeutic applications. Targeted gene therapy for systemic delivery of proteins by intramuscular injection of expression plasmids provides a convenient method of studying the therapeutic functions of specific proteins. In addition, owing to prolonged and sustained serum target protein levels, intramuscular gene delivery methods have a potential to replace periodic injections of therapeutic proteins with shorter systemic half-lives such as erythropoietin in anemia and GCSF in severe congenital neutropenia or aplastic anemia. Delivery of heterologous genes such as TIMP-4 into skeletal muscles has been shown to provide sustained serum levels of TIMP-4 protein, which can effectively inhibit the Wilms' tumor growth and induced arthritis. These results suggest that intramuscular DNA injection is a useful method of systemic delivering cytokines, growth regulators, and other serum proteins. The naked DNA-mediated gene therapy will eventually enter the market with much more promising future due to: (a) its being non-toxic and having fewer side effects than virus-mediated gene therapy; and (b) simple and inexpensive large scale manufacture.

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Chapter 2

Heparin and Gene Microarrays as a New Pharmacodynamic Tool

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Traditionally the effect of a drug on a tissue is measured by methods that are either limited in scope or in information. Individual protein and mRNA levels measured by Northern and Western blotting or by Immunohistochemistry, while demonstrating an effect and providing some mechanistic description provide information only on those targeted molecules. Likewise, morphometric analyses of tissues examine the gross tissue response, but provide little insight into the underlying mechanisms responsible for the present state of the tissue. Gene micro-arrays allow the screening of thousands of mRNA expression levels in a single sample. This technique therefore yields data that is both tissue wide in scope and mechanistic in nature. This becomes especially useful when the drug being examined has effects beyond that of the desired effect. In this chapter, Heparin and its ability to inhibit intimal hyperplasia following arterial injury will serve as an example of a disease and intervention where gene microarrays can be applied as a new pharmacodynamic tool. Heparin's function as an anticoagulant is well described, but it is also known to possess numerous other functions that are less well understood. It is both the pleiotropic effects of Heparin and the complexity of vascular disease that make this an ideal situation for the use of gene microarrays to monitor the efficacy of an drug delivery method.

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In the development of new delivery methods from bioactive compounds, the goal is to achieve the optimum balance between the efficacious concentrations at the site of action and the undesirable effects in non-target tissues. For example, the local delivery of antiproliferative agents by coated stents achieves effective drug concentrations at the diseased site while avoiding undesirable effects in non-target tissues. Traditionally the effect of an agent on a tissue has been measured using methods that either measure the resultant effect on the tissue as a whole such as histological methods or count on the ability of a surrogate marker to indicate efficacy. These methods are limited in either scope or in information. Gene microarrays allow for the monitoring of thousands of mRNA levels from a single sample. Such a massively parallel method yields a vast amount of data while also allowing the user to adjust the scope from tissue wide to individual cell isolates. Therefore gene microarrays are an excellent tool for pharmacodynamic comparisons between delivery methods when a broad scope in the data is desired.

This chapter will discuss the advantages of this method for pharmacodynamic comparisons in the case of Heparin in prevention of intimal hyperplasia following arterial injury. There are two reasons for this choice as an example. The arterial response to vascular injury is a complex series of processes resulting in the eventual loss of lumen diameter. There is the initial dilation of the artery, the loss of endothelium, thrombus formation, the recruitment of inflammatory cells, and the eventual remodeling of the vessel. The result of these processes is the migration and proliferation of the medial smooth muscle cells to form a neointima and a loss of some or all of the lumenal area. Thus, to fully assess a compound's ability to block these processes, a method with a broad scope is required. Unfortunately, as one increases this scope, mechanistic information is often lost. For instance, morphometric measurements of histological samples can indicate whether a delivery method was effective in blocking neoinimal formation, but does not yield information on which individual processes in the vascular response to injury were altered to achieve the result. Only a method with the broad scope of gene microarrays is able to simultaneously monitor the various processes of the response to vascular injury.

Heparin, endogenous analogs, and related compounds mediate arterial injury to an extent seen with virtually no other compound, and yet it is still not clear how this effect is achieved. Heparin regulates leukocyte adhesion and diapedesis (1, 2), extracellular matrix remodeling (3-6), growth factor displacement (7), cytokine production (8-10), induction of cell proliferation, protein kinase expression (11, 12), endothelium-dependent relaxation and vasodilation (13, 14), and re-endothelialization (15). Because there are multiple areas in which Heparin has been shown to have an effect, the exact mechanism by which it blocks intimal hyperplasia has yet to be elucidated. Research to date has largely focused on individual areas of the vascular response to injury (i.e. leukocyte adhesion) and hoped that the data acquired will extrapolate to the bulk tissue response. Unfortunately this has often not been the case.

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Continuous intravenous administration of Heparin almost completely blocks intimal hyperplasia in simple animal models of vascular injury, but this effect has yet to be demonstrated in higher animals and clinically (16, 17). Whether this loss of effect arises from a problem of dosing or from changes in species remains unclear despite over twenty years of investigation. One likely explanation is that as the dosing method changes, some or all of the multiple effects of Heparin are lost. Thus, Heparin is ideal compound for pharmacodynamic analysis using gene microarrays, because they allow the monitoring of diverse families of genes on a tissue wide scope.

This chapter will begin with a summary of the processes of the vascular response to injury and of Heparin's role in inhibiting vascular proliferative disease. Then the use of gene microarrays to examine Heparin's use as an intervention will be introduced.

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Heparin and the Arterial Response to Injury

The vascular response to injury is a complex and multifaceted set of processes that culminate in the loss of loss lumen diameter. Heparin and its analogs have been shown to effect the various aspects of these processes as well as inhibiting the ultimate lumen loss. The extent of interplay between the individual processes and the responsibility of each for the global tissue response is still unclear. Even the history of the discovery of Heparin's antiproliferative ability is an example of how a pleiotrophic drug requires a broader tool to assess its effects. It is both Heparin's diverse effects and the complexity of the disease that make this an ideal example of how gene microarrays can be used as a powerful pharmacodynamic tool.

The Vascular Response to Injury

The relationship between the smooth muscle cells and the endothelial cells is the defining measure of the health of blood vessel. The overlying endothelial cells serve as a thromboresistant protective barrier to the vascular smooth muscle cells. The mechanical health of the vessel is maintained by the vascular smooth muscle cells, which control vascular tone and luminal diameter through their synchronous contraction or relaxation (18). Upon injury these contractile cells undergo a phenotypic switch to synthetic cells. Now, the cells in the vascular media are stimulated and smooth muscle cells will begin proliferating (19-23). Likewise, migration of vascular smooth muscle cells from the media to the intima occurs leading to myointimal thickening. This set of events is coupled with alterations in local lipid metabolism, thrombosis and haemostasis and leukocyte/monocyte adhesion to generate an obstructive occlusion over a period of 40-60 years (24, 25). Severe injuries such that of percutaneous transluminal coronary angioplasty can accelerate this process to within months.

Ross has described the earliest events in atheriosclerosis as the adhesion of platelets and/or leukocytes to luminal surfaces with dysfunctional or missing endothelium(24, 25). This is followed by the secretion of chemotactic and mitogenic factors for vascular smooth muscle cells. Purely mitogenic growth factors for vascular smooth muscle cells associated with this proliferation include fibroblast growth factors –1, and –2, epidermal growth factor, insulin-like growth factor-1, Heparin-binding growth factor, and under certain conditions transforming growth factor β (18). Platelet derived growth factor has been shown be both chemotactic and mitogenic for vascular smooth muscle cells. The presentation of these growth factors activates a cascade of response elements which often includes amplification signals, such as the secretion of monocyte chemotactic protein-1 by macrophages which is chemotactic for both macrophages and vascular smooth muscle cells.

It is important to note that the proliferating cells in the intima have undergone a phenotypic modulation to a synthetic phenotype. These cells show a loss of myofilaments and an increase in the endoplasmic reticulum and Golgi apparatus (26-28). The cells also show a decrease in α-actin (29-31), isoform changes in myosin (32), tropomyosin content (33), decrease in desmin and increase in tenacin expression (34), an increased response to mitogens, and an increased level collagens and extracellular matrix proteins (35). This modulation is reversible under specific conditions including treatment with Heparin.

Heparin's Antiproliferative Ability

As the normal vessel is able to maintain a healthy ratio of intima:media, it is clear there are natural products which promote this quiescent state and inhibit the proliferation and migration of smooth muscle cells. These natural products include retinoic acid, interferons, TGF-β, and Heparin (18). In the mid seventies it was known that thrombin was a potential mitogen for different types of mesenchymal cells. It had also been observed that deendothialization of an artery led to the adherrance of platelets and the eventually massive myointimal thickening. The possibility that this injury activated the clotting cascade and promoted the generation of fibrin and thrombin lead to the hypothesis that anticoagulants which activated antithrombin might reduce intimal hyperplasia. Continuous intravenous administration of Heparin was found to markedly decrease the intimal thickening and smooth muscle cell proliferation while not affecting the number of adherrant platelets in deendothelialized arteries (16, 18). This has been demonstrated in several models of restenosis.

The next step was to determine whether the anticoagulant properties of Heparin correlated to the antiproliferative effects. When separated on a antithrombin III affinity column, it was found that non-anticoagulative fractions

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of Heparin were as potent antiproliferative agents as the those containing the anticoagulant potency in vitro (36). Furthermore, other glycosaminoglycans, including dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid, show no antiproliferative effect (37).

Heparin has become the standard for inhibition of smooth muscle cells in culture, and has been shown to inhibit intimal hyperplasia when continuous intravenous dosing is employed in rabbits and rats. There is also increasing evidence that the cell surface heparan sulfate proteoglycans play a major role in the control of arterial health. Though identified as a potential antiproliferative agent because of its anticoagulant properties, it is now clear that its growth inhibitory activity is separate from its anticoagulant activity.

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Structure and Function

The derivation of this novel activity becomes even more complicated when one examines the relationship between structure and function. Heparin is a highly sulfated heteromeric polymer of alternating D-glucosamine and uronic acid sugars linked 1-4. The D-glucosamine is either N-acetylated or N-sulfated. The uronic acid residues are either D-glucuronic acid or L-iduronic acid. Heparin is sold commercially as a polydisperse mixture containing varying degrees of sulfation. The antiproliferative activity can vary greatly between different preparations, and there is no correlation between the anticoagulant activity units and a lot's antiproliferative ability. Some basic structure-function relationships have been determined. The antiproliferative properties require at least a pentamer (38). While O-sulfation is required for both the antiproliferative and anticoagulant effects, N-sulfation is required only for anticoagulant activity (38). Increases in negative charge increase the antiproliferative activity. Oversulfation at the O-position increases the antiproliferative activity, but decarboxylation decreases the same activity (39). As a strong demonstration of the lack structural correlation between the two activities, 3-O-sulfation on the internal D-glucosamine of the pentasaccharide which binds antithrombin III is minimally antiproliferative and this sequence is often missing in highly antiproliferative preparations (39). It therefore appears that Heparin's activity may not lie simply in the presence of a single sachharide sequence. Instead there may be multiple sequences responsible in whole or in part for the antiproliferative activity with each possessing their own individual effect.

Localization of Action

A final aspect of Heparin function that complicates our understanding of it's activity is the localization of action. Heparin is bound by vascular smooth muscle cells and seems to be internalized by a receptor-mediated endocytosis. There are approximately 105 specific binding sites for Heparin on vascular smooth muscle cells. These sites are protease-sensitive and have a K_d of 10^{-9} M (40, 41). Growth arrested vascular smooth muscle cells have been demonstrated to bind eight times more Heparin and to be 50 to 100 times more sensitive the antiproliferative effect of Heparin than vascular smooth muscle cells growing expontially. Upon binding the Heparin appears to be rapidly internalized with a $t_{1/2}$ of 15 minutes (41). Following the uptake the Heparin is degraded and secreted.

Recently developed Heparin resistant cells have allowed the elucidation of further aspects of the binding, internalization, and degradation of Heparin. The binding affinities and the number of sites appeared to be the same for both Heparin sensitive and resistant vascular smooth muscle cells. While internalization of the Heparin is the same in both cell lines in the initial phase, the Heparin-sensitive cells increase their uptake and the Heparin insensitive cells cease to internalize Heparin after prolonged exposure to the drug. Likewise, degradation of the internalized Heparin appears to follow the kinetics of the uptake (42).

Together, these data suggest that changes in preparation, injury model, and delivery method, which alter the duration, concentration, and saccharide sequences of a Heparin treatment, may effect the intervention's effectiveness in injured arteries. Furthermore, these changes may not be evident at the gross tissue level, as similar growth inhibition is possible with differing preparations. What is required to analyze this type of intervention is a massively parallel method which can examine a target tissue and monitor the various areas of activity.

Mechanisms of Heparin Antiproliferative Effect

There are several distinctly different processes encompassing the arterial response to injury. These processes include leukocyte adhesion, cellular migration, and cell proliferation. Heparin has been shown to demonstrate a positive effect on each one, with no single effect having been identified as responsible for the antiproliferative activity. Here we shall introduce several of these effects to demonstrate the advantage of gene microarrays is assessing Heparin delivery methods.

Leukocyte Adhesion

One of the earliest events in the response is the adhesion of inflammatory cells to the site of endothelial injury. This process consists of four distinct steps: (i) the leucocyte rolling along the endothelial surface mediated by

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selectins; (ii) adhesion triggered by cytokines; (iii) strong adhesion and (iv) chemotactic migration of the leucocyte . Heparin's role in leucocyte adhesion may be three fold. First it may inhibit the expression of the cytokines which induce the initial adhesion . Second, it may bind to those cytokines preventing their attachment to the luminal surface (43). Finally, Heparin may block the function of enzymes which facilitate the penetration of the leucocyte within the vessel wall (1).

Heparin and heparan sulfate have been demonstrated to bind numerous molecules involved in leukocyte recruitment including interferon-γ, interleukin-1, tumor necrosis factor, and other chemokines. Human microvascular endothelium cells show an increase in the signal transducer and activator of transcription protein, as well as monocyte chemotactic peptide-1 when stimulated by interferon-γ. Douglas and coworkers have demonstrated that this stimulation is blocked when the interferon -γ is mixed with Heparin (44, 45).

There is also evidence Heparin may directly effect the adhesion and diapedesis of leukocytes at sites of inflammation. The selectins are a group of glycoproteins which may be responsible for the initial adhesion of leukocytes to sites of inflammation. Significantly, Heparin has been shown to bind to both the P- and L- selectins which may prevent their presentation on the surface of cells at the site of inflammation (46, 47). Lider and coworkers have proposed that the enzyme heparanase may play a critical role in the penetration of lymphocytes into the cell well, and that Heparin may inhibit this enzyme's function (1).

Extracellular Matrix

The extracellular matrix is implicated in the inflammatory response of cells to injury, both in the ability of cells to migrate and the in the response to mitogens. It is believed that cell surface proteoglycans play a major role in cellular adhesion to the extracellular matrix. Heparin has been shown to inhibit the growth and adhesion of Balb/c-3T3 fibroblasts and vascular smooth muscle cells to collagen (48, 49). This may occur through a displacement of the heparan sulfate proteoglycans from the Heparin binding sites on collagen. In a similar manner, platelet derived growth factor stimulated cells show an increase of both thrombospondin as well as its incorporation of in the cell surface, but the only the cell surface incorporation is inhibited by the presence of Heparin. Heparin has also been demonstrated to induce a shape change in vascular smooth muscle cells, which may play a role in the adhesion of molecules to the extracellular matrix (50). More recently it has been shown that the plasminogen activators, may play a role in the remodeling of the extracellular matrix. Heparin inhibits the expression of both of these enzymes (51-53).

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Growth Factor Displacement

Heparin is known to bind several growth factors including platelet derived growth factor, acidic and basic fibroblast growth factor, and epidermal growth factor (54-56). Furthermore, the binding of heparan sulfate and basic fibroblast growth factor is required for the formation of a stable complex on the cell surface or in the extracellular matrix (57, 58). This lead to the hypothesis that the antiproliferative function of Heparin is through a displacement of these growth factors from their target receptors. Especially since it has been demostrated that Heparin can displace basic fibroblast growth factor from the cell surface and extracellular matrix, which leads to its rapid clearance by the liver and kidney (18).

Many studies have since challenged the validity of that hypothesis. Heparin has been shown to inhibit the growth of cells which grown in serum which has been depleted of Heparin-binding proteins by passage through a sephadex-Heparin column (59). When cells are grown in a large excess of platelet derived growth factor there is no decrease in Heparin's antiproliferative ability (59). Also Heparin is able to inhibit cell growth in cells grown in platelet derived growth factor free serum (60). Finally, Heparin can still inhibit growth 12-14 hours after the addition of serum, far greater than the time it takes vascular smooth muscle cells to respond to platelet derived growth factor (18).

Heparin Specific Signaling Pathway

Finally, there has been extensive research into intercellular mechanisms by which Heparin may function. The first evidence of the pathway used by Heparin is seen in the inability of Heparin to inhibit epidermal growth factor stimulated cell growth. Epidermal growth factor activates the cyclic-AMP and Protein Kinase A pathways, whereas platelet derived growth factor, basic fibroblast growth factor, and phorbol esters activate the Protein Kinase C pathway (18). Heparin is also able to suppress growth in cells stimulated with PMA, a Protein Kinase C pathway specific mitogen. Pukac and coworkers have shown that this inhibition of the Protein Kinase C pathway is downstream or parallel to Protein Kinase. C(61). Herbert and coworkers demonstrated the administration of antisense molecules to Protein Kinase C-α selectively prevented the antiproliferative effect of Heparin.

A second central player in the signaling of Heparin antiproliferative activity is mitogen activated protein kinase. Heparin has been demonstrated to inhibit the phosphorylation of mitogen activated protein kinase when cells are stimulated by platelet derived growth factor and serum, but not in response to epidermal growth factor (62). It have also been shown that the p42 isotype of mitogen activated protein kinase is largely responsible for the response to injury and that mitogen activated protein kinase kinase-1 is responsible for 80-90% of the activation of mitogen activated protein kinase. Heparin have been shown to inhibit both of these activities (63, 64).

Protein Kinase C and Mitogen activated protein kinase represent two pathways identified with numerous cellular processes. Heparin's activity extends beyond these pathways to many others. For instance, our laboratory recently linked Heparin to a down regulation of the EphB2 receptor that correlated with a decrease in vascular smooth cell growth (65). These pathways are presented as an example of Heparin's effect on signal transduction.

Thus, just as the site of action and required structure for antiproliferative activity remain unclear, the mechanisms by which Heparin acts are diverse and interrelated. Under certain conditions Heparin may displace relevant growth factors, while under different conditions its effect may be limited to a specific kinase pathway. If one was to choose a surrogate marker for Heparin's antiproliferative activity there are numerous options with ample scientific evidence to support their use. For instance one could choose a specific cytokine to monitor, and could design an experiement that correlates Heparin's effect with a change in that protein. However, if the preparation of Heparin, the delivery kinetics, or the animal model are changed this effect may be lost without a loss in antiproliferative activity as the mechanism behind the activity is altered. Gene microarrays provide a broad screen of messenger RNA levels so that instead of using a single mRNA to monitor an activity, a few hundred or thousand genes can be used.

Gene Microarrays as a Pharmacodynamic Tool

In most cases when a tissue becomes diseased there is a drastic change in the mRNA and proteins being expressed. This is clearly seen in the vascular response to injury as described above. One of the simplest ways to compare microarray data is to directly compare the intensity values of two datasets. If two tissue are in the same state, for example the healthy quiescent state, then the mRNA expression profile should be similar in both tissues. If instead, one tissue has greatly altered its mRNA expression in response to a disease state, that linearity is lost. This is seen clearly in Figure 1, which compares the mRNA expression values of an uninjured artery to a balloon denuded artery. A tight linear correlation is seen between the two normal arteries. This indicates that the majority of mRNA's are being expressed in similar proportions in both tissues. When an injured artery is compared to the normal artery a loss in the linearity is seen, indicating that the two tissues are expressing mRNA's at different levels, and are in different states.

It is important to remember that in doing this type of analysis, we are comparing hundreds or thousands of mRNA's from numerous families of genes to determine which tissues are behaving in a similar manner. We are no longer limited to a single marker, hoping it will be definitive. For instance, Figure 2

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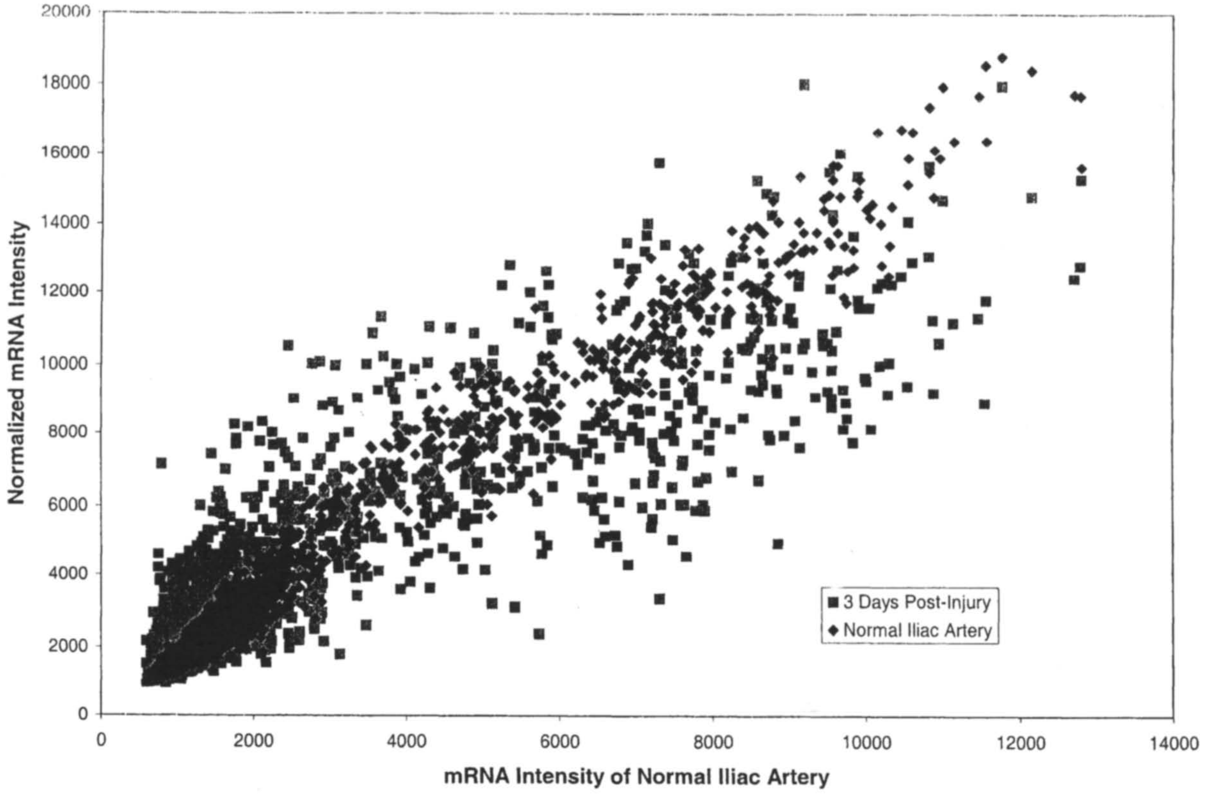

Figure 1. Comparison of mRNA levels of normal and injured rabbit iliac arteries as measured with Research Genetics Human GeneFilters™.

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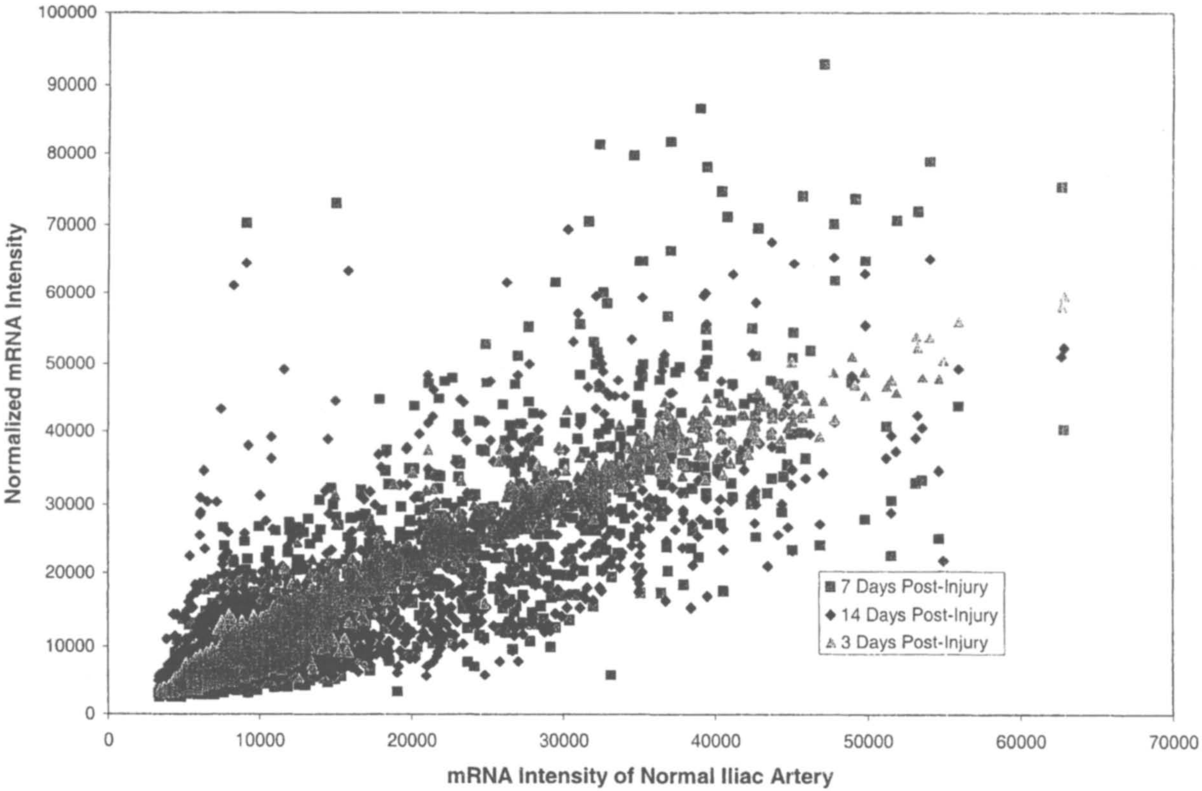

Figure 2. Comparison of mRNA levels of injured rabbit iliac arteries at 3, 7, and 14 days after injury to mRNA levels seen at 3 days after injury as measured with Research Genetics Human GeneFilters™.

In Advances in Controlled Drug Delivery; Dinh, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

demonstrates that the vascular response to injury changes greatly over time. There is a tight correlation between tissues taken at 3 days after injury, but this no longer true when comparing mRNA profiles from tissues from 7 and 14 days post-injury to the 3 days post injury sample. These differences are expected as there are several temporal phases of the vascular response to injury. If a single marker identified as important at three days was chosen, it may indicate a decrease in the need for intervention at 7 or 14 days when in fact the response is simply entered a new phase. By using the microarrays, it becomes clear that the tissue is not only not in a normal state, but has progressed in the response to injury. Furthermore, having identified perturbations in mRNA expression specific to each individual state, data mining techniques can be used to investigate the mechanisms behind the response.

The previous two figures have demonstrated the usefulness of this technique to vascular disease, but it can just as easily be applied to assessing a drug's efficacy. Figure 3 demonstrates that administration of a continuous intravenous dose of Heparin can greatly alter the mRNA expression of an injured artery. In this case the linear response represents a similarity to an injured artery at three days. When Heparin is administered continuously over those three days we see a loss in that linearity indicative of a shift in the injured artery's state.

Finally, Figure 4 presents the comparison of these two samples to a normal artery. Of course the goal of any intervention in vascular disease is to return the vessel to its original healthy state. In our case that is represented as a specific mRNA profile. In Figure 4, it is clear that the treatment of an injured artery with Heparin lead to a mRNA profile at 14 days post injury that is more similar to the normal artery's profile than that of injury alone. Thus, in a single experiment and a single figure we are able to assess the efficacy of a specific Heparin treatment.

Again, now that profiles of mRNA expression have been determined for each state, one can begin to determine families of genes being effected by vascular injury and Heparin treatment. The results of this analysis will be published separately, but in brief the effects seen when the altered mRNA's are examined are as expected from the literature. Decreases in the mRNA levels of members of the mitogen activated protein kinase and Protein Kinase C pathways were altered. An attenuation of adhesion molecules, cytokines, and other mRNA's seen in inflamed tissue was observed. Finally, changes in mRNA encoding cytoskeletal components and growth factors were also seen. This diverse set of mRNA's is indicative of the broadness of the scope of this technique.

Conclusion

Before the advent of gene microarrays, the ability to assess the state of a tissue was limited to methods that either lacked scope (i.e. immunoblotting) or lacked detail (i.e. gross tissue morphometry). With gene microarrays we are able

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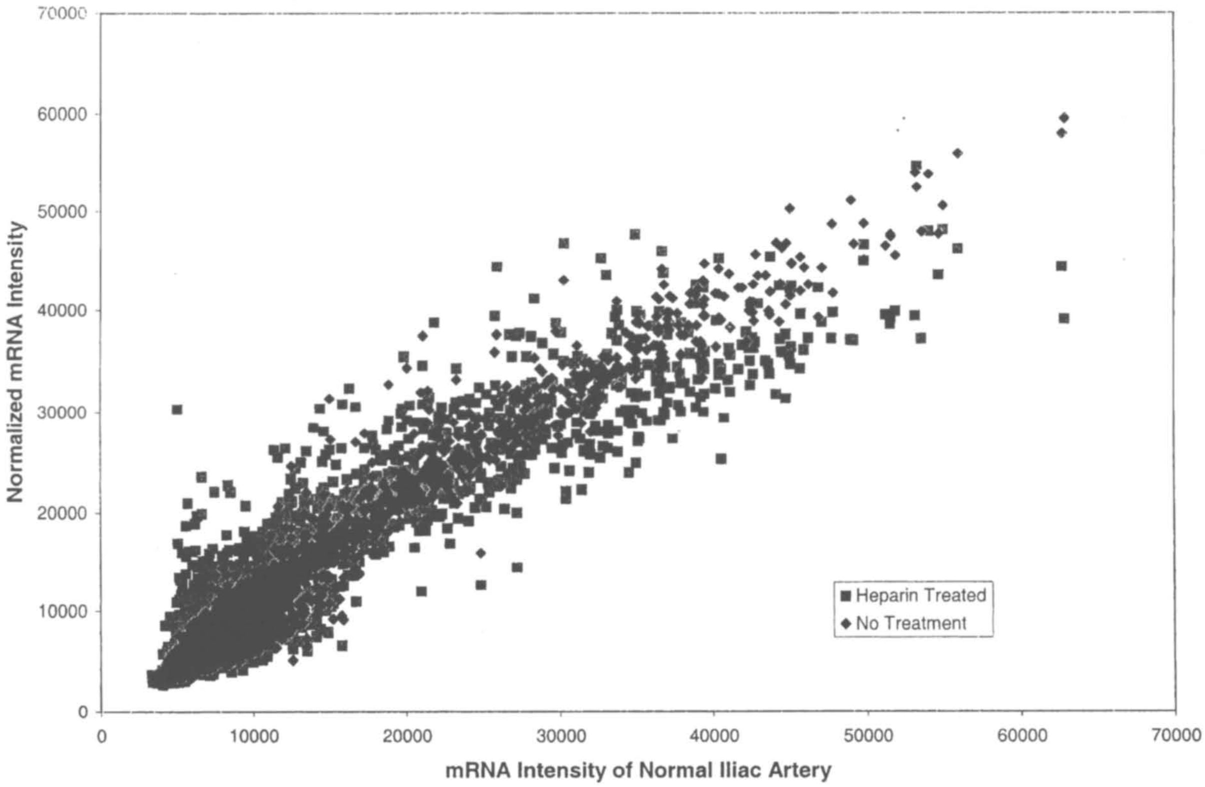

Figure 3. Comparison of mRNA levels of injured rabbit iliac arteries at 3 days after injury with and without continuous intravenous Heparin treatment as measured with Research Genetics Human GeneFilters™.

to monitor changes in thousands of mRNA from a single sample. That sample can be a whole tissue homogenate, a microdissected sample, or an *in vitro* culture. Pairwise analysis of the instensities of these mRNA expression levels allows the determination of whether the two tissue are expressing mRNA in proportion to each other. This in effect is determining whether the tissues are undergoing the same processes or are in the same state. In certain cases where a drug's pharmacodynamic activity is easily measured, this technique may appear too robust. However, Heparin is an excellent example of a drug whose pharmacodynamic measurement is often considered very straightforward, an elevation in APTT while in fact Heparin's activities extend far beyond that of anticoagulation. Gene microarrays are an excellent tool for assessing these extended effects. Likewise in complicated disease states like vascular disease, where numerous families of genes are turned on and off throughout the course of the disease, gene microarrays can again be used to monitor all aspects of a disease's progression. As the ability to analyze the vast amount of data generated increases, so will our ability to design drug formulations and delivery methods to target diseases.

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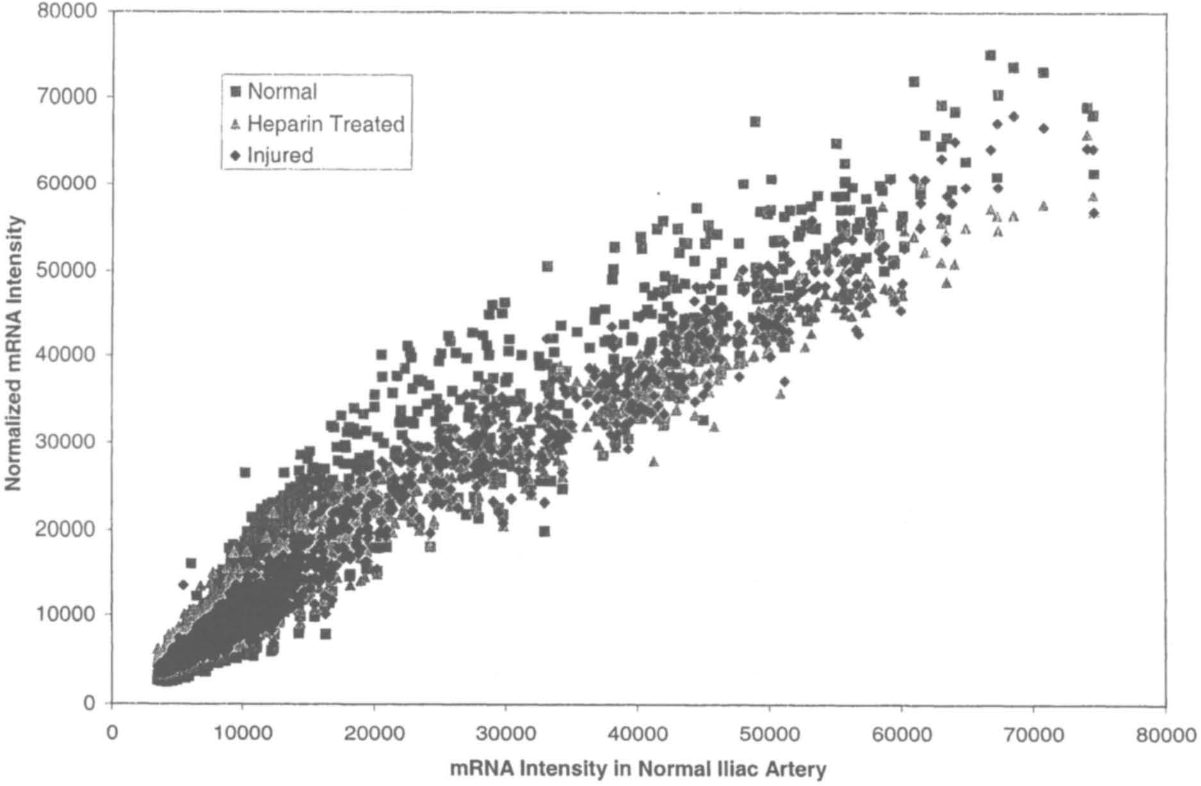

Figure 4. Comparison of mRNA levels of injured rabbit iliac arteries at 14 days after injury with and without continuous intravenous Heparin treatment to mRNA levels of normal iliac arteries as measured with Research Genetics Human GeneFilters™.

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Chapter 3

Oral Heparin Prevents Neointimal Growth Following Vascular Injury: Implications for Potential Clinical Use

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Background: Heparin is the archetypical modulator of vascular repair following arterial injury in animal models. In these models, heparin delivered either as a continuous intravenous infusion or via frequent subcutaneous dosing, inhibits neointimal hyperplasia after either balloon injury or stent implantation. However, use of subcutaneous heparin following human percutaneous intervention has failed to prevent restenosis. It may be that these failures arise from a need for more frequent dosing regimens in man. Recently, the drug delivery agent Sodium *N*-caprylate (SNAC) has been found to facilitate gastric absorption of heparin raising the possibility of convenient frequent dosing. **Methods and Results:** To investigate the effects of orally delivered heparin on neointimal hyperplasia following varying forms of arterial injury, New Zealand White rabbits underwent iliac artery balloon dilatation. In half of the animals, endovascular stents were implanted and heparin delivered through different methods. Arteries were harvested at 14 days and neointimal area was assessed using computer-aided morphometry. Following balloon injury, both intravenous (0.3 mg/kg/hr) and

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oral heparin (90 mg/kg BID) inhibited neointimal growth (0.11±0.02 and 0.09±0.07 mm^2 respectively vs. control 0.16±0.06 mm^2, $p<0.05$). Following stent implantation, intravenous administration of heparin (0.3 mg/kg/hr) inhibited neointimal growth (0.35±0.05 mm^2 vs. control 0.45±0.09 mm^2, $p<0.05$), but oral heparin was not effective at either 90 mg/kg BID or 180 mg/kg BID (0.48±0.04 and 0.49±0.08 mm^2, p=NS vs. control). However, the same dose given more frequently (120 mg/kg TID) was effective (0.40±0.10 mm^2, $p<0.05$ vs. control). **Conclusions:** Oral heparin effectively inhibits neointimal growth following vascular injury. Stented arteries require higher and more frequent dosing for efficacy than that required after simple balloon dilatation. These data indicate that oral heparin may be an effective method of suppressing restenosis, and that differences in type of vascular injury should be considered in the design of drug delivery.

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INTRODUCTION

Since Gruntzig's landmark publication in 1979 introducing angioplasty for the treatment of coronary artery stenoses[1], the use of percutaneous coronary intervention has grown widely with an explosive increase in the number of patients and the complexity of lesions treated. Yet even in this first publication, the major limitation of this procedure was evident in that 19% of patients undergoing successful initial procedures suffered restenosis at follow-up. Larger scale studies over the next decade revealed the incidence of restenosis to be 30-40%[2]. The search for effective therapies against restenosis has been the major thrust of catheterization laboratory-based research over the last two decades. In the early 1990's, intracoronary stents were introduced with the BENESTENT[3] and STRESS[4] trials, and were the first therapy to effectively reduce restenosis rates from approximately 30% to 20%. Based largely on these pivotal studies, stents are now used in the majority of percutaneous coronary interventions. However, in-stent restenosis has emerged as a particularly difficult disease process to treat. Therefore, the search for adjunctive therapies to treat restenosis remains a high priority in the interventional community.

Heparin is the archetypical modulator of vascular repair after vascular injury in a number of animal models. It has long been known that heparin inhibits SMC proliferation and neointimal hyperplasia independent of its anticoagulant properties[5,6]. Clowes and Clowes[7] first demonstrated that heparin inhibited proliferation within the media (maximal at four days) and intima (maximal at seven days), as well as SMC migration from the media into the

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intima after balloon injury in a rat carotid model. In a subsequent study[8], they reported that when heparin was delivered for a week, there were prolonged effects on neointimal area at two and four weeks post-injury. They also demonstrated that heparin started 48 to 96 hours after injury was ineffective at reducing neointimal growth, suggesting that heparin's inhibitory influence in the balloon injury model is greatest early in the post-injury period, presumably prior to cells entering the S-phase.

However, human trials of subcutaneous heparin in the treatment of restenosis following angioplasty have failed[9]. A potential explanation of this difference is suggested by data from animal studies that demonstrate that efficacy of heparin in preventing neointimal growth is dependent on the type of vascular injury imposed[10] and the duration and frequency of heparin dosage[11]. These studies suggest that the type of vascular injury (i.e. stent implantation vs. balloon denudation) and pharmacokinetic properties of agents must be understood when designing strategies against restenosis. More specifically, these data suggest that human studies of heparin following angioplasty may have been compromised by inadequate dosing interval and duration of administration.

Heparin delivered via the oral route is poorly absorbed from the intestinal tract and rapidly degraded. Therefore, heparin has traditionally been administered via either intravenous or frequent subcutaneous administration making administration on an outpatient basis difficult. The molecule Sodium *N*-caprylate[12] (SNAC) has been found to allow gastric absorption of heparin[12,13]. Utilizing SNAC, the studies described in this chapter were undertaken to examine the efficacy of oral heparin against neointimal growth following vascular injury.

The studies described in this chapter demonstrate an inhibitory effect of oral heparin on neointimal growth following vascular injury after balloon denudation with or without stenting in a rabbit iliac artery model[14]. Compared to balloon injured arteries alone, arteries from stented animals required a more frequent and higher drug dose to inhibit neointimal growth[14]. An oral form of heparin may make frequent administration more practical and therefore, more effective in the suppression of restenosis in humans. Also, these data and others demonstrate that vascular injury imposed by stents is fundamentally different than that from balloon dilatation and these differences must be understood for the appropriate design of anti-proliferative strategies.

METHODS

Surgical Procedure

As described previously[14], New Zealand White Rabbits (Covance Products, Denver, PA), weighing three to four kg, were used for the studies described in this chapter. They were housed individually in steel mesh cages and fed rabbit chow and water ad libitum. All animal care and procedures were in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care and the National Institutes of Health. After achieving anesthesia with intramuscular ketamine (Aveco Co., Fort Dodge, IA) 35 mg/kg and xylazine (Miles Inc., Shawnee Mission, KS) 15 mg/kg, the femoral arteries were exposed and ligated bilaterally. Femoral arteriotomies were performed and a three French balloon catheter (Baxter HealthCare Corp., Irvine, CA) was passed in retrograde fashion into the abdominal aorta and withdrawn in the inflated state three times to denude the iliac artery endothelium. In the first series of experiments, balloon injury alone was performed (n= 25 animals, 43 arteries). In the second (n=32 animals, 51 arteries), following balloon injury, a seven mm corrugated ring stainless steel endovascular stent mounted on a three mm angioplasty balloon (Advanced Cardiovascular Systems/Guidant, Santa Clara, CA) was passed in retrograde fashion into each iliac artery and deployed with a 15-second inflation to a pressure of eight atmospheres. Standard anticoagulant heparin (100 U/kg, Elkin-Sinn inc., Cherry Hill, NJ) was injected as a single intravenous bolus prior to deployment of all of the stents. To reduce the incidence of subacute stent thrombosis, all animals received aspirin (Sigma Chemical Co., St. Louis, MO) in their drinking water (0.07 mg/mL) to achieve an approximate dose of 5 mg/kg/day. ASA was started one day prior to the procedure and lasting for the duration of the experiment.

Sodium heparin USP (Hepar Industries, Franklin, OH) was delivered from subcutaneously implanted osmotic minipumps (Alza Corp. Palo Alto, CA) through a catheter within the femoral vein at 0.3mg/kg/hr (n= 8 animals in the balloon-injured group, n= 4 animals in the stent group). Using 150 mg/kg SNAC, heparin (Hepar Industries, Franklin, OH) was delivered via oral gavage at 90 mg/kg BID (1 ml/kg animal body weight) in the balloon-injured group (n= 4 animals). In the stented group, heparin was delivered via oral gavage at either 90 mg/kg BID (n= 3 animals), 180 mg/kg BID (n= 5 animals) and at 120 mg/kg TID (n= 5 animals). In 5 animals SNAC alone at 150 mg/kg BID was given to balloon-injured animals to assess the potential effects of SNAC itself.

Tissue Processing

All animals were sacrificed 14 days after vascular injury. Anesthesia was administered as above, the caudal vena cava was opened, and pressure perfusion performed with Ringer's lactate solution (300 cc) through left ventricular puncture, followed by 0.4% para-formaldehyde for 10 minutes at 100 mm Hg pressure. The iliac arteries were excised and placed in a solution of 0.4% para-formaldehyde. Specimens were embedded in methyl methacrylate mixed with *n*-butyl methacrylate (Sigma Chemical Co., St. Louis, MO). Five micron sections were cut using a tungsten-carbide knife (Delaware Diamond Knives, Inc, Wilmington, DE). Stented specimens were oriented to the proximal and distal

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ends and sections were taken at three points along the stent, including each end and the middle, to reduce sampling error.

Dose Determination

In an initial study of 4 rabbits, heparin levels were determined to be 0.005±0.003 mg/ml 2 days after initiation of intravenous heparin therapy via osmotic minipump (0.03 mg/kg/hr). An acute time course study was then performed. Two anesthetized rabbits underwent orogastric lavage to remove gastric contents. Oral heparin was then delivered (with 150 mg/kg SNAC) via orogastric tube at 36 mg/kg and 90 mg/kg. Plasma heparin levels were determined at baseline and 15, 30, 60, 120, 180, and 240 minutes. Blood was sampled through the femoral vein via an 18 g i.v. catheter into a 3.8% sodium citrate vacutainer. Heparin levels were measured using the Heptest and Heptest Hi reagent kits (Sigma Diagnostics, St. Louis, MO) and an Amelung KC1 Clot Timer. This assay measures the inhibition of clotting by heparin in the presence of a known amount of Factor Xa. Coefficients of absorption and elimination were calculated to allow estimation of plasma concentrations at varying doses and dose intervals (Figure 1). An initial oral dose of 90 mg/kg BID was chosen for the study to achieve an average plasma concentration of 0.004 mg/ml.

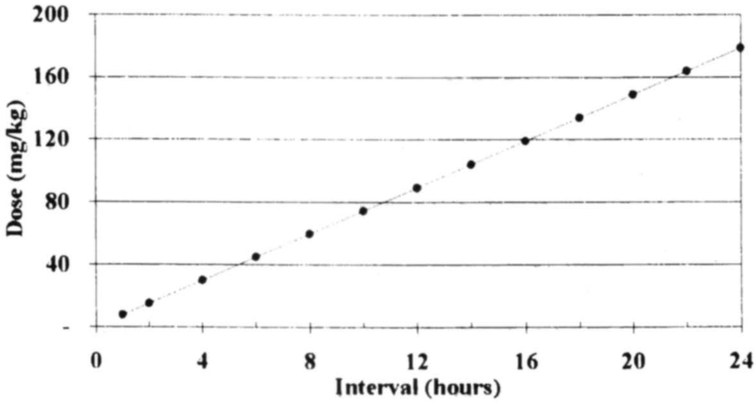

Figure 1. Calculated dosing requirements at different intervals to maintain an average plasma concentration of 0.004 mg/ml. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)

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Histology and Statistical Analysis

Tissue and cells structures were identified in histological sections by staining with Verhoeff's tissue elastin stain. Neointimal and medial cross-sectional areas were measured by computer-assisted digital planimetry. For stented arteries, values from proximal, mid, and distal sections were averaged. All data are presented as mean±SD. Statistical comparisons were performed with a one-way ANOVA utilizing the least significant difference methods for multiple comparisons versus control. Values of $p<0.05$ were considered significant.

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RESULTS

Animals underwent either superficial balloon endothelial denudation, or deep chronic injury by balloon denudation followed by stent implantation. Heparin was delivered via 2 routes; the standard intravenous route which has been shown in the past to effectively inhibit neointimal hyperplasia in both models of injury, or through the newer oral route.

Balloon Injury

At 14 days after balloon injury, heparin suppressed neointimal growth when delivered intravenously or via oral gavage at 90 mg/kg PO BID (Table 1, Figures 2 and 3) compared to control. The oral drug delivery agent SNAC 150 mg/kg PO BID delivered without heparin had no effect compared to control.

Stent Injury

Following stent-induced injury, a different pattern was seen. Intravenous heparin effectively suppressed neointimal growth, but oral heparin at 90 mg/kg PO BID, or twice this dose, had no effect when compared to control arteries. In contrast, the same total large dose (360 mg/kg/day) was effective when delivered in three daily (120 mg/kg PO TID), rather than two daily doses (180 mg/kg PO BID) (Table 2, Figures 4 and 5).

DISCUSSION

These data demonstrate that oral heparin is effective against neointimal growth following either superficial balloon injury or deep chronic injury seen

Table 1. Intimal areas in balloon-injured arteries. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)

Treatment	*Intima mm^2 (mean±SD)*
Control	0.16±0.02
Sham	0.16±0.06
0.3 mg/kg IV	0.11±0.05*
90 mg/kg PO BID	0.09±0.07*
* $p<0.05$	

Table 2. Intimal area in stented arteries. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)

Treatment	*Intima mm^2 (mean±SD)*
Control	0.45±0.09
90 mg/kg PO BID	0.48±0.04
0.3 mg/kg IV	0.35±0.05*
180 mg/kg PO BID	0.49±0.08
120 mg/kg PO TID	0.40±0.10*
* $p<0.05$	

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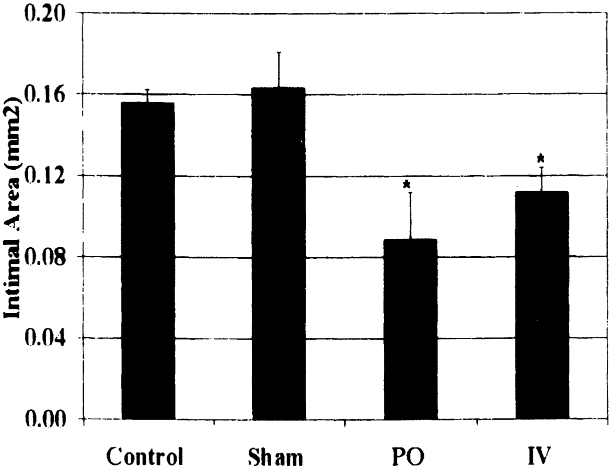

*Figure 2. Bar graph showing 14 day neointimal areas in balloon-injured arteries for different treatment groups. *$p < 0.05$. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)*

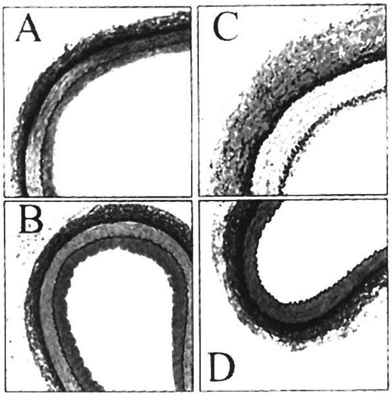

Figure 3. Photomicrographs (20×) of balloon-injured arteries. A – Control. B – Sham. C – Intravenous heparin. D – Oral heparin 90 mg/kg BID. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)

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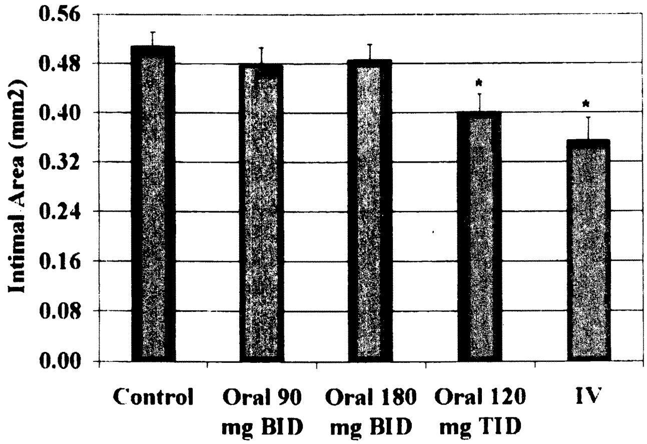

*Figure 4. Bar Graph showing 14 day neointimal areas in stented arteries for different treatment groups. *p < 0.05. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)*

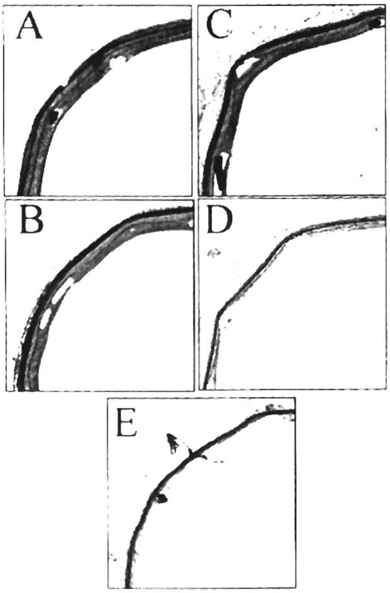

Figure 5. Photomicrographs (20×) of stented arteries. A – Control. B – Oral heparin 90 mg/kg BID. C – Oral heparin 180 mg/kg BID. D – Oral heparin 180 mg/kg BID. E – Intravenous heparin. (Adapted with permission from reference 14. Copyright 2001 American Heart Association)

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with stenting[14]. Also, these data demonstrate that stented arteries required both a larger dose and more frequent delivery to be effective[14]. These data suggest that oral heparin may offer an effective strategy against restenosis in humans and lends further support to the notion that there are important differences between the injury associated with balloon endothelial denudation and that associated with the chronic injury of stent implantation.

Prior Data from Animal and Human Studies

Animal studies- Heparin is the archetypical modulator of vascular repair and is a potent inhibitor of smooth muscle cell proliferation in vitro[15] and of neointimal growth in a number of animal models of vascular injury[5,7,16,17]. Postulated mechanisms, independent of its anticoagulant activity[6], include inhibition of nuclear transcription factors[18], change in growth factor activity and receptor binding[19], alteration of extracellular matrix production[20] by smooth muscle cells, direct inhibition of smooth muscle cell proliferation and migration[21], and an as an anti-inflammatory agent[22-25]. Several studies have demonstrated that the inhibitory potential of heparin is intimately related to the type of vascular injury and on the dosage regimen. After balloon injury in a rabbit iliac artery model, heparin delivered either as a continuous intravenous infusion or by twice daily (BID) subcutaneous dosing inhibits neointimal growth, but heparin delivered less frequently is not effective and may actually enhance neointimal growth[11]. Additionally, Rogers et al.[10] showed that stented rabbit iliac arteries require administration of heparin for 14 days to effectively inhibit neointimal hyperplasia. However, in balloon-injured arteries, a short 3 day course of heparin was as effective as drug administration for the 14 day course of the experiment. Prior studies of the inflammatory response after vascular injury offer potential explanations for these findings. Following balloon injury in a rabbit iliac artery model, immunohistologic examination reveals only an early and transient neutrophil infiltration[25]. However, after the chronic and more profound injury associated with implantation of a stent, the early neutrophil infiltration is much more intense, and is accompanied by a later, more sustained accumulation of macrophages within the neointima[10]. Intravenous heparin, has been shown to reduce the number of infiltrative leukocytes with a simultaneous suppression of SMC proliferation and neointimal growth in balloon-injured and stented arteries[10,25].

The studies presented in this chapter substantiate these earlier findings in that stented arteries, with their larger and more chronic burden of inflammatory cells, require a larger dose of heparin compared to balloon injured arteries[14]. In addition, these data further support the notion that frequency of heparin administration is important for its efficacy as we have demonstrated that

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the same total dose of oral heparin was only effective against neointimal hyperplasia in stented arteries when delivered more frequently[14].

Human studies- Given the wealth of data supporting heparin as a modulator of vascular repair in animal models of vascular injury, there has been longstanding interest in its use clinically. However, published clinical trials of heparin in patients undergoing angioplasty have been uniformly disappointing. For example, Ellis et al.[9] randomized 416 patients undergoing balloon angioplasty to either 18-24 hours of unfractionated heparin or dextrose and found no difference in late (180±81 days) angiographic follow-up. Due to their more stable pharmacokinetic and pharmacodynamic profile, low molecular weight heparins have been studied in a number of studies of patients undergoing balloon angioplasty. Despite these pharmacokinetic and pharmacodynamics advantages, these agents have not been found to reduce angiographic restenosis rates[26-28]. The prior animal data and the data presented in this chapter showing the need for more frequent heparin dosing to achieve efficacy against neointimal growth following vascular injury offers an explanation for these paradoxical findings and suggest that these human studies may have suffered from an insufficient dosing regimen.

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Mechanism of Action of SNAC

SNAC is a synthetic compound with a molecular weight of 310 daltons. The exact mechanism of action of SNAC in facilitating gastric absorption of heparin has not been fully elucidated. It has been postulated that SNAC forms a non-covalent bond with heparin that makes possible gastric absorption[12,13]. In animal models, oral heparin has been shown to be an anticoagulant, elevating aPTTs and has been shown to prevent deep venous thrombosis in a rat model[29,30]. In humans, oral heparin has been shown to raise aPTTs in a dose dependent fashion[31]. The preparation was tolerated well and did not result in significant side effects or toxicity. The studies described in this chapter demonstrate efficacy of oral heparin against neointimal growth after vascular injury in a rabbit iliac artery model[14].

Conclusion

The studies described in this chapter demonstrate that, in a rabbit iliac artery model, oral heparin is an effective agent against neointimal hyperplasia following superficial balloon induced injury and the more chronic deep injury associated with stent implantation[14]. Also, these studies add to prior observations showing that there are differences in the vascular biologic response to injury between balloon injury and the injury associated with a chronic indwelling stent. The more intense and prolonged inflammation associated with stenting appears to require a larger and more frequent dosing schedule compared to simple balloon endothelial denudation[14].

The development of a safe and effective method for oral delivery of heparin offers the possibility of more frequent, prolonged, and convenient delivery of heparin to patients. It therefore can achieve a different pharmacokinetic and pharmacodynamic profile than that of subcutaneous or intravenously injected heparin which may prove effective in the prevention of restenosis. The use of these compounds in human trials of restenosis prevention may validate the longstanding notion that heparin is indeed a potent modulator of vascular repair.

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Chapter 4

Peptide Vector-Mediated Drug Delivery to the Brain

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Delivery of drugs to the nervous system remains a challenge despite remarkable advances in our understanding of the mechanisms involved in the development of the brain diseases and the actions of neuroactive agents. Drug accessibility to the central nervous system is limited by the blood-brain barrier that is formed by brain capillary endothelial cells. Since the endothelial cells are connected to each other by tight junctions, compounds must cross the membranes of the cells to enter the brain from the bloodstream. Therefore, hydrophilic compounds cannot cross the barrier in the absence of specific mechanisms such as membrane transporters or endocytosis. The BBB is, therefore, the major obstacle to drugs that are potentially useful for combating diseases affecting the CNS. Extensive efforts have been made to develop CNS drug delivery strategies in order to enhance delivery of therapeutic molecules across the BBB. One approach to overcoming the BBB is to transport drugs via peptide vector-mediated strategy.

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As a consequence of the growing aging population, many neurodegenerative diseases, cancer, and infections of the brain will become more prevalent. One of the foremost problems in drug delivery to the brain is the presence of the blood-brain barrier (BBB), which prevents transport of most drugs from the vasculature into the brain parenchyma. While a wide variety of neuropharmaceutical drugs are presently available, few possess the required physicochemical properties that render them suitable for the treatment of central nervous system (CNS) diseases.

The endothelium of the central nervous system vasculature shows structural differences compared to that of most organs, featuring tight junctions between the endothelial cells and abolishing into the internal environment of the brain any aqueous paracellular pathways for water-soluble agents (1). The function of the BBB is dynamically regulated by various cells present at the level of the BBB, comprising astrocytes, neurons, and pericytes (2). The task of the BBB is therefore to guarantee the transfer of special nutrients to the brain and to exclude the transfer of most small polar molecules and macromolecules from cerebrovascular circulation to the brain.

Progress in pharmacology and neurosciences resulted in greater knowledge of CNS diseases and of potential therapies, but also created the need to develop new strategies to improve drug delivery to the brain. Transport kinetic, metabolic, cellular and molecular studies have given a major impetus to a better understanding of how the BBB functions, and how this can be exploited in brain delivery terms (3-5). The most important factors determining to what extent a molecule will be delivered from blood into CNS are lipid solubility, molecular mass, and charge. These structural characteristics allow for passive penetration of the molecules across the BBB. The "classical" neuropharmaceutical agents in the market or in clinical trials are typically less than 600 Da molecular weight with a sufficient degree of solubility. Therefore, based both on their lipid solubilities and molecular masses, the passage of neuropharmaceuticals that fall into the category of peptide-based drugs (neuroactive peptides, neurotrophic factors, cytokines, monoclonal antibodies) and nucleotide-based agents such as oligonucleotides and genes will be impeded by the BBB.

Anatomy and Physiology of the BBB

The continuous layer of cerebrovascular endothelium is commonly termed as the blood-brain barrier (6,7). It possesses complex tight junctions and a low number of pinocytotic vesicles in contrast to the epithelium in other parts of the body. Due to these characteristics, the BBB limits the delivery of hydrophilic drugs and electrolytes to the CNS. Ions can only enter the brain by passing between the endothelial cells via the tight junctions, while non-electrolytes pass across the endothelial cells, their permeability being proportional to their lipid solubility. Since any drug available to the brain parenchyma has to pass through this barrier, the efficacy of any drug therapy based on systemic delivery is dependent greatly on the permeability of the barrier to the drug.

The cerebrovascular endothelial layer is leakier in some circumventricular organs such as the area postrema or the median eminence. Some molecules may also reach the choroid plexus and enter the cerebrospinal fluid (CSF) by transependymal transport (8,9). However, the surface area available for transport by these routes is much smaller than the area of the BBB. Hence the BBB is the most important barrier to drug delivery.

In addition to its passive properties the BBB in some cases, can actively block the entry of some endogenous or exogenous compounds into the brain. The endothelial cells contain a large number of mitochondria, indicating a high metabolic activity. Endothelial cells of human capillary blood vessels at the blood-brain and other blood-tissue barrier sites express P-glycoprotein (P-gp) (10). The up-regulation of the P-glycoprotein efflux pump may prevent the entry of various drugs including some anticancer agents such as doxorubicin.

Finally the BBB has an enzymatic component. In fact, the BBB has also been called an "enzymatic barrier," because it has the ability to inactivate vasoactive peptides and drugs such as leukotrienes (11).

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Endogenous BBB Transport Systems

Permeation of compounds across the BBB has long been believed to be dependent on their lipophilicity. However, although some molecules such as doxorubicin and vinblastine are highly lipophilic, the apparent permeation of these drugs across the BBB was unexpectedly low (12-14). This relationship indicates that lipophilicity is not necessarily a useful predictor of transfer into the brain. Furthermore, most nutrients in the circulating blood, in spite of having low lipophilicity, are well known to be efficiently taken up into the brain. These apparently contradictory observations can be ascribed to the existence of multiple mechanisms of drug transport through the BBB.

Most drugs pass between blood and brain extracellular fluid (ECF) via the lipid membranes of the endothelial cells because the capillary wall lacks water-filled channels of suitable size for entry by aqueous diffusion.

Small lipophilic drugs enter the brain easily, penetrating the lipid membrane of the endothelial cells (15). Passive diffusion of a drug correlates with its blood/brain concentration gradient and liposolubility but it is inversely related to extent of ionization and molecular weight (12). For organic molecules, transfer across the BBB takes place mainly through the endothelial cells. There are two likely processes for this movement: carrier-mediated transport, which displays characteristics of a carrier-mediated process (stereospecificity, self-saturation and competitive inhibition) and endocytosis mechanisms (13, 16-18). The carrier transfer at the BBB can be either by facilitated diffusion that simply expedites the movement of molecules equally well on either direction, by the influx pumps that actively extract compounds from blood to brain, or by the efflux pump (P-glycoprotein), that actively transports solutes from brain to blood.

Receptor-mediated transport systems work via endosomal structures in which substrates are taken up by energy-requiring systems into endosomal sacks and then transported from the cytosolic compartment of the endothelial cells to the extracellular space of the brain or in the reverse direction (19). The compounds must use pinocytic vesicles and adsorptive-mediated or receptor-mediated transcytosis. Well-known mechanisms of receptor-mediated pathway are those for transferrin (20) or insulin (21).

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Strategies for Overcoming the BBB

To overcome the limited access of drugs to the brain, different methods have been developed (22-25). These approaches can mainly be divided into three categories:

1) Surgical Delivery

A direct way to circumvent the BBB is to surgically deliver the drug to the brain parenchyma. This can take the form of intracranial injection, or implantation of infusion pumps, and genetically modified cells.

Intraventricular drug infusion involves placing a catheter into the cerebrospinal fluid of the ventricles and relies on drug diffusion from local depot sites to the brain parenchyma. Unfortunately, diffusion is a poor mode of drug delivery to the brain and the limited volume of distribution in tissue of therapeutic drugs that can be achieved by administration into brain limits the therapeutic utility of this route of delivery. Implantable drug pump can deliver drugs in a sustained manner at predetermined rates but is applicable only to those drugs that are stable in the buffer solution. It has been used to deliver narcotic analgesics for pain control, dopaminergic agonists for Parkinson's disease, and anti-neoplastic agents for brain tumors (26). Problems associated with implantable drug pumps include catheter infection mechanical failure and dislodging of needle.

Gene transfer of autologous cells has been proposed to treat malignant brain tumors and to prevent neuronal degeneration. An interesting strategy is to introduce a suicide gene coding for the herpes simplex virus type 1 thymidine kinase (HSV1-TK) into tumor cells followed by ganciclovir treatment (27).

Cells that express HSV1-TK become sensitive to ganciclovir, which is non-toxic to normal cells.

2) Temporary Disruption of the Barrier

Since the tight junctions act as a tightly bound wall, one way to "sneak" therapeutic drugs past the BBB is by disrupting it. Disruption of the BBB can be achieved by infusion of hypertonic solutions. Biologically active agents such as bradykinin and angiotensin peptides are also capable under some circumstances of disrupting the BBB, suggesting that they may play a role in modulating the behavior of the BBB.

The osmotic effect of hypertonic solutions has been used to reversibly and temporarily open the BBB to deliver the drugs to the brain. Hypertonic solutions of mannitol shrink the endothelial cells lining the brain and widen the tight junctions making the BBB leaky. Patients with different types of tumors in the brain have been treated with this approach (28-30).

Mediators of the inflammatory response, such as leukotrienes, can induce transient vascular leakage and temporarily increase the permeability of blood vessels (31). Normal brain capillaries resist the vasoactive effect of these compounds due to the presence of an "enzymatic barrier". The BBB contains a unique enzyme [gamma] - glutamyl transpeptidase, not present in the peripheral circulation that inactivates leukotrienes C4 to D4 and E4 to F4. Injured capillaries, or capillaries in brain tumors lose this ability to inactivate leukotrienes and show an increased permeability after leukotriene C4 infusions. This fact was used to increase the permeability of blood tumor barrier in rats with RG-2 gliomas (32).

3) Drug Modification

Various techniques have been used to enhance the penetration of a drug into the CNS. Some of these techniques are briefly described below.

a) Lipophilic drug delivery

Lipophilic drugs penetrate the BBB easily by transcellular diffusion. Most psychopharmaceutic drugs fall into this category (33). Synthesizing a lipophilic analog of an otherwise impermeable drug is a logical first attempt to target the drug to the brain. Another variation on the theme is the use of "prodrugs". The CNS- active drug is bound to a chemical moiety that renders the conjugate lipophilic. These prodrugs may be hydrolyzed in the target tissue by esterases or oxidized as in a redox-based chemical delivery system, to trap the drug in the

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brain. In such a redox-based system, 1-methyl-1,4-dihydronicotinate has been successfully attached to various drugs such as estrogen, zivudine (AZT), ganciclovir, anti-epileptic drugs such as valproate and phenytoin among others, and has been found to increase their brain penetration (34,35).

b) Receptor/Carrier Systems

Another approach for delivering drugs to the brain involves specialized transport systems that exist in the brain. The presence of these carrier/receptor systems, and groups of related substrates has been documented (36). To take advantage of the facilitated transport, a drug is engineered to mimic the substrate, or linked to that particular substrate. For instance, the delivery of L-dopa for Parkinson's disease uses a large neutral amino-acid transporter such as phenylalanine as a carrier (37). Carried across the BBB, L-dopa is then decarboxylated to yield dopamine.

The same principle has also been used in the delivery of the so-called chimeric peptides. A chimeric peptide contains the pharmacologically active peptide bound covalently to another peptide that has an associated receptor system in the cerebral endothelium. A highly studied receptor-mediated transport vector is the monoclonal antibody OX26, which recognizes an external epitope of the transferrin receptor (38). OX26 is semi-brain specific, since it targets the brain and liver more than the heart, lung and kidney. This antibody has been used for the delivery of peptides such as vasoactive intestinal peptide (VIP) (39), brain derived neurotrophic factor (40) and nucleic acid analogues (41). The peptide of choice is coupled to the delivery vector via disulfide bonds that can be cleaved in the parenchyma of the brain.

Other vectors that could be employed for this purpose are cationic proteins that can undergo absorptive-mediated transcytosis through the BBB. The most widely studied vector of this type is cationized rat-albumin (42). This vector is also semi-brain specific, showing a significant uptake in the kidney, while showing very little or no uptake in the spleen, heart and liver.

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PEPTIDE-MEDIATED STRATEGY

The discovery that small peptide vectors can transport drugs across complex biological membranes has opened up new possibilities in biomedical research. Peptide vectors such as SynB vectors, Penetratin, and TAT have been used successfully to deliver biologically active substances inside live cells (43).

The drug that lacks transport at the BBB is coupled to a vector that has access to the brain.

SynB vectors are short linear peptides derived from naural peptides called protegrins that are able to cross cell membranes without any cytolytic effect. These vectors have been used to enhance the brain uptake of a wide range of molecules including small molecules, peptides, and proteins.

In one study, we assessed the efficacy of SynB vectors to enhance the brain uptake of the anticancer agent doxorubicin. Doxorubicin was coupled to SynB vectors via a chemical linker (succinate) and its ability to cross the BBB was studied using in situ cerebral perfusion in rats and mice (14,44). Coupling of doxorubicin to SynB vectors significantly enhances its brain uptake in all the gray areas assessed and without compromising BBB integrity (Table 1). The amount of vectorised doxorubicin that was delivered to the brain parenchyma was about 20 to 50-fold higher than for free doxorubicin, depending on the vector used. Interestingly, we have observed that SynB vectorised doxorubicin bypasses the P-gp that has been shown to be present at the luminal site of the endothelial cells of the BBB (45). The ability of SynB vectors to enhance the brain uptake of doxorubicin was also assessed after intravenous injection of vectorised doxorubicin into mice. The tissue and plasma distribution of doxorubicin were dramatically modified when the drug was vectorised. The brain concentrations were higher for vectorised doxorubicin compared to that of free doxorubicin (14). Interestingly, vectorised doxorubicin shows significantly lower levels in the heart; strongly suggesting that cardiotoxicity - the main side effect of doxorubicin - could be reduced using this strategy.

Table 1: Brain uptake in different brain areas

Brain Areas	*Free doxo (µl/g/sec)*	*Dox-SynB1 (µl/g/sec)*
Frontal Cortex	0.18	1.6
Mesencephal	0.38	1.8
Occipital Cortex	0.71	1.7
Parietal Cortx	0.47	2.7
Thalamus	0.69	2.2
Hippocampus	0.3	2.2
Striatum	0.15	1.5

Note: Transfer coefficients (Kin) for free and vectorised doxorubicin uptake in rat brain areas after perfusion with buffer. Each value represents a mean of 4 animals.

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In order to assess the broad potential of this approach, we have coupled an antibiotic, benzylpenicillin (B-Pc), to SynB vector and measured its brain uptake using the *in situ* brain perfusion in mice. Antibiotics must cross the BBB to be suitable for use in the treatment of severe cerebral infections such as bacterial meningitis. We have demonstrated that coupling of B-Pc to SynB vectors improves its penetration across the BBB in a significant manner and that the vectorised B-Pc was distributed in all gray areas.

The use of SynB vectors has also been successfully applied to brain delivery of peptide drugs. In a pharmacological application focused on pain management, the brain uptake of an enkephalin analogue was enhanced several 100-fold after vectorisation. Importantly, systemic administration of the vectorised enkephalin led to a dramatic enhancement of its analgesic effect in mice compared to that of free enkephalin (Rousselle et al, unpublished results).

The mechanism by which the vectorised drugs cross the BBB appears to occur via adsorptive-mediated endocytosis. First, the transport of vectorised doxorubicin is a saturable mechanism and the observed Km values in the micromolar range. Second, the brain transport does not involve a chiral receptor since no difference in brain uptake can be seen between L-SynB vector and its enantio form D-SynB. Finally, the transport process in vivo can be inhibited by positively charged polymers such as poly (L-lysine) and protamine. All these data suggest that the transport is via adsorptive-mediated endocytosis (44).

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Conclusion

The blood-brain barrier poses a formidable obstacle to drug therapy of the central nervous system. As new drugs for neurological disorders are discovered, new delivery techniques will have to be developed in concert to overcome this transport barrier. As rapid advances in cell and molecular biology lead to a proliferation of potent macromolecules that cannot be effectively delivered to the brain by conventional means, continuing refinement of the new delivery methods will be essential to realizing the potential of these macromolecular drugs. The use of peptide-vectors in which non-transportable drug is chemically linked to a transportable peptide-vector has already shown promising results and should be given serious considerations.

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Chapter 5

Systemic and Topical Lung Delivery Using the AER_x System

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Pulmonary drug delivery offers many advantages over conventional routes of delivery, including a very large surface area for absorption and direct access to the blood stream via a non-invasive delivery route. Patient convenience and compliance may also be improved through the elimination of needles associated with subcutaneous injection. As a result, research and development of systems for systemic delivery has intensified over recent years. Topical pulmonary delivery using MDIs and DPIs has a long history. Development of improved aerosol delivery systems will increase efficiency and reduce variability as well as target the drug to a specific region of the lung to reduce side effects. Efficient systemic delivery requires a tight control of the emitted dose, the particle size distribution, and the patient's inhalation flow rate. The latter two parameters are also important to target delivery to different regions of the lungs; e.g., the central airways or the alveoli. The AER_x® Pulmonary Delivery System can be tailored to deliver drugs either systemically or topically at high delivery efficiency. The results of both in-vivo and in-vitro studies using the AER_x system for both systemic and topical delivery are presented.

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Advantages of Advanced Pulmonary Drug Delivery

Pulmonary drug delivery has a long history of relatively low efficiency and relatively high dose variability, which has only allowed the development of topical delivery of therapeutic drugs, with few side effects, and a relatively large therapeutic window (*1,2*). However, pulmonary drug delivery has the potential to offer many advantages over conventional delivery routes. It provides an opportunity to administer medications non-invasively in a reproducible and efficient manner. The lung contains a very large surface area for absorption of a drug-containing aerosol; the average adult has a total surface area of approximately 100 square meters, about the size of a singles tennis court. This large surface area offers near direct access to the blood stream providing the possibility of fast onset for a wide variety of pharmaceuticals including both small molecules (*3-5*) as well as protein drugs (*6-8*).

Pulmonary drug delivery also provides an opportunity to improve compliance with existing therapies utilizing more conventional delivery routes, such as injection. It also enables the targeting of drugs either topically to the lung tissues themselves, or systemically to the blood stream.

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Requirements for Pulmonary Drug Delivery

Pulmonary drug delivery, either for topical administration or for systemic delivery, requires the reproducible and efficient delivery of an inhaled aerosol to the lung. In both cases, efficient dosing and reduction of side effects require deposition of the aerosol in the lung itself, and not the mouth or oropharynx. In the case of systemic delivery, the target is the peripheral lung or alveoli. In this region, the resistance to transport to the blood stream is minimized. The absorption rate is maximized, bioavailability is improved, and mucociliary clearance is avoided.

Targeting the lung, and indeed, specific regions of the lung, requires precise control over the aerosol particle size distribution, the inhalation flow rate, which determines the particle velocity, as well as the point during the inhalation at which the aerosol is generated and taken into the lungs. The region of the respiratory tract in which the aerosol is deposited, depends, to a large extent, on the momentum of the aerosol particle as it moves through the lung. The particle momentum is equal to the product of its mass and its velocity. As the momentum increases, so does the probability of deposition higher in the respiratory tract. This is illustrated in Figures 1 and 2 taken from Byron (*9*) and Morrow and Yu (*10*), respectively. It is evident from Figure 1 that the ideal

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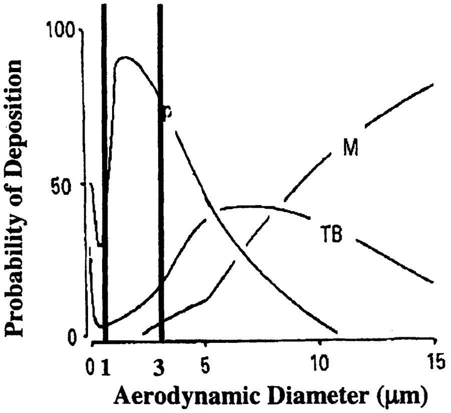

Figure 1: Aerosol Deposition as a Function of Median Particle Size, P = Pulmonary (deep lung), TB = Tracheo-Bronchial, M = Mouth. (Adapted with permission from reference 9. Copyright 1986, Wiley-Liss.)

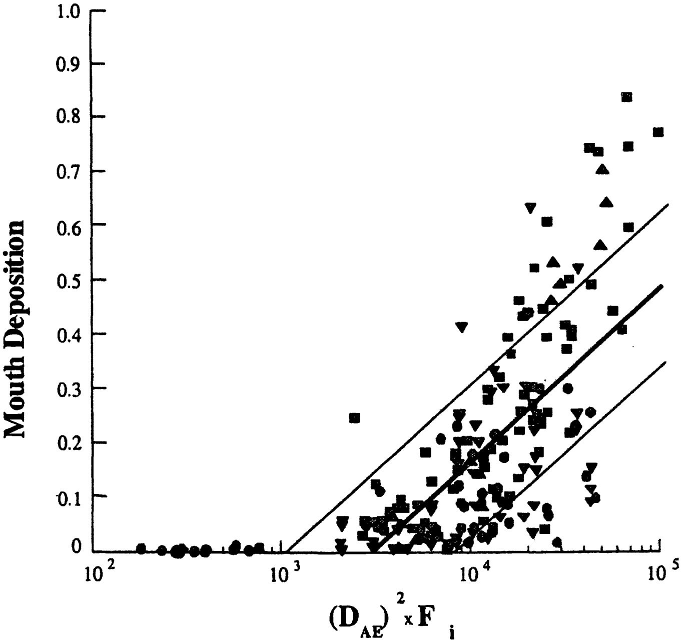

Figure 2: Probability of Aerosol Deposition in the Mouth as a Function of a Momentum Parameter, D_{AE} = Aerodynamic Diameter, F_i = Inhalation Flow Rate. The different symbols represent results from 4 different studies as described in reference 10. (Reproduced with permission from reference 10. Copyright 1985.)

aerodynamic particle size for deposition in the lung is between approximately 1 and 3 microns, inhaled at a controlled flow rate. Figure 2 illustrates that mouth deposition can be nearly eliminated, thus increasing the efficiency of delivery, by controlling the inhalation flow rate (F_i) and the particle size (D_{AE}). The volume of air inhaled prior to aerosol generation has also been shown to be an important factor in determining the regional deposition in the lungs (*11*).

Conventional Aerosol Delivery Systems

There are several aerosol delivery systems that are currently available on the market. The metered dose inhaler (MDI) and the dry powder inhaler (DPI) are typically not capable of delivering drugs to the peripheral lung, and as shown below, can have a relatively high level of variability. These types of devices attempt to control the product-related variables: drug concentration and particle size distribution. However, they do nothing to control the patient-related variables: inhalation flow rate and patient education and compliance. Additionally, the MDI does not control the point in the inhalation at which the device is actuated. As an example an MDI is used to deliver asthma drugs topically to the lung with relatively low efficiency and non-specific targeting of the delivery. This can result in side effects due to non-targeted aerosol, that is, drug that deposits in the mouth and throat rather than the lung. It can also result in a high variability both between doses in a single patient, as well as among patients. The data in Table I, from Borgström, et.al. (*12*), were gathered for topical delivery with a Turbuhaler®, a type of DPI, and with an MDI, under realistic dosing conditions. The patients were dosed in the clinic four times at weekly intervals. They were trained in the clinic on the first day of dosing only. Between the first day of dosing, and each of the subsequent analysis days, they were told to practice once per day at home.

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Table I: Conventional MDI and DPI Variability In-Vivo

	Turbuhaler	*PMDI*
Lung Deposition (% of nominal dose)	20	6
Within Subject (%RSD)	33	65
Between Subject (%RSD)	28	62

Source: Reproduced with permission from reference 12. Copyright 1996, Interpharm.

The results indicated the high degree of variability in these systems when used under realistic home-use conditions. A large portion of that variability is due to differences in the way patients use their inhalers. In-vitro results from the same study indicate a within-device variability for Turbuhaler of 18.2 %RSD and for pMDI of 6.4 %RSD over ten actuations. Therefore, the data in Table I show that patient compliance and correct usage of an inhalation device is a critical ingredient for accurate and efficient delivery of drugs via the lung.

A third type of commercially available delivery system is the jet nebulizer. This system delivers aerosolized liquid drug formulations by tidal (normal) breathing, over an extended period of time, rather than as a single-inhalation bolus delivery. The necessity of controlling inhalation flow rate and coordinating device actuation are reduced or eliminated. However, the rate of delivery tends to be very low, necessitating long delivery times. This results in inconvenience to the patient and reduced patient compliance.

In summary, these conventional aerosol delivery systems are limited to drugs that can be delivered topically, and are effective at a wide range of doses with few side effects due to over or under dosing, or improper dosing. Therefore, to meet the requirements for systemic delivery, or for topical delivery of drugs that have a narrow therapeutic window, a new approach is needed.

Advanced Pulmonary Drug Delivery

Aradigm's AERx® Pulmonary Delivery System attempts to control most of the important variables which determine reproducible and efficient dose to lung, as well as which control the region of deposition within the lung. These variables include the patient-related variables such as the inhalation flow rate, the point in the inhalation at which the aerosol is actuated, and the total inhaled volume, as well as system-related variables such as the emitted dose and the aerosol aerodynamic particle size distribution. The system also provides continued education to the patient to maintain compliance with therapy.

The AERx system initiates aerosol delivery at a preprogrammed inhalation flow rate and inhaled volume and delivers the aerosol dose over a fixed period of time (*13*), thus controlling the particle velocity and inhaled volume at the point of actuation. After actuation, the system continues to guide the patient, using red, green and yellow lights on the top of the device, to inhale completely and within a defined flow rate range. The patient is thus "trained" to use the system correctly each time a drug is administered.

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In vitro Performance

The AERx system has demonstrated reproducible in-vitro performance across a large number of developmental programs and over a long period of time. Figure 3 shows the results of device release testing for a clinical study using an AERx system prototype. The mean emitted dose (n=2) at each of three flow rates (total of 6 measurements per device) are shown for 85 devices. The mean emitted dose for all devices was approximately 100% of the target emitted dose, with a %RSD of 4%. The data indicate that there is no difference in emitted dose across the range of flow rates measured. Figure 4 shows the results of 12 months of stability testing on a single lot of AERx dosage forms stored at 5°C and 25°C / 40%RH. The Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) were calculated from a least squares fit by non-linear regression of data gathered using an Anderson Cascade Impactor. The data indicate that the particle size distribution generated using the AERx system is almost monodisperse, and remains consistent over at least twelve months of storage at 5°C and typical room temperature conditions.

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In vivo Performance

AERx clinical data, assessed by gamma scintigraphy, are similarly consistent (*14-18*). A recent clinical scintigraphy study (*14*) resulted in a mean emitted dose of 65% of the dose loaded into the dosage form with a %RSD of 10% within subjects, and 13% among subjects. This compares very favorably with the data in Table I for conventional systems, and provides the consistency necessary to deliver drugs systemically.

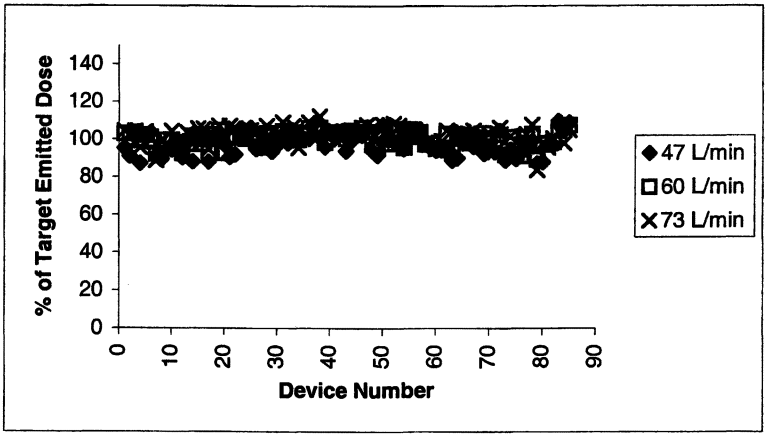

Figure 3: In-Vitro AERx Emitted Dose is Consistent Across 85 Devices and 3 Inhalation Flow Rates.

Aradigm's AERx pulmonary delivery system has produced several important results when compared to other modes of delivery. In clinical trials pulmonary delivery has been shown to have an equivalent time to peak serum morphine concentration as intravenous injection (*5*), a faster reduction of glucose levels with insulin as compared with subcutaneous injection (*8*), a 90% reduction in dosing time, when compared to a conventional jet nebulizer (*15*), and the potential to deliver high amounts of a drug in a few inhalations (*18*).

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Pulmonary Delivery for Systemically Active Drugs

Small Molecules

Pulmonary delivery can be used for systemically active drugs that are either small molecules or large protein drugs. Morphine, an example of a small molecule drug, has been shown to be safe when delivered using the AERx System in several clinical trials (*3-5*). In a cross-over study in six healthy volunteers, comparing AERx administration of 4.4 mg delivered over 2.1 minutes on three separate occasions and intravenous (IV) infusion administration of 2.0 mg and 4.0 mg delivered over 3 minutes on separate occasions (*4*). The results, Figure 5 and Table II, show that the two routes of administration are equivalent in terms of the pharmacokinetic profiles. The bioavailability of inhaled morphine was approximately 100% relative to intravenous infusion, with similar intersubject variability in AUC for both routes (%RSD<30%).

Table II: AERx vs. IV Morphine PK Data

	AERx (4.4 mg)		*IV (4.0 mg)*		*IV (2.0 mg)*	
	Mean	*SD*	***Mean***	*SD*	***Mean***	*SD*
Cmax (ng/nL)	**109**	86	**273**	114	**165**	22
Tmax (min)	**2.7**	0.8	**3.2**	0.8	**3.0**	0.9
AUC(∞) (ng*min/mL)	**2795**	892	**2317**	657	**1434**	118

Intravenous morphine was administered over 3 minutes and pulmonary morphine was administered over a mean of 2.1 minutes.

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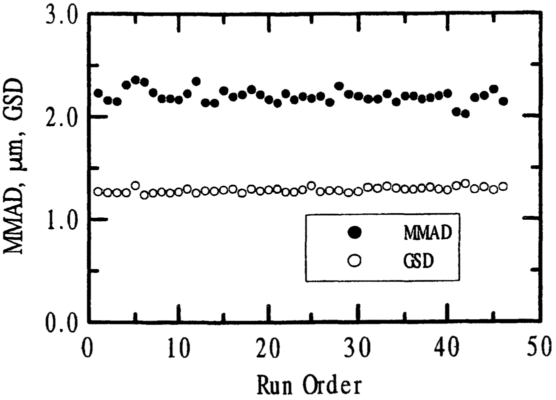

Figure 4: AERx Particle Size Distribution is Consistent Over 12 Months Using Dosage Forms Stored at 5°C and 25°C / 40%RH (MMAD = Mass Median Aerodynamic Diameter, GSD = Geometric Standard Deviation).

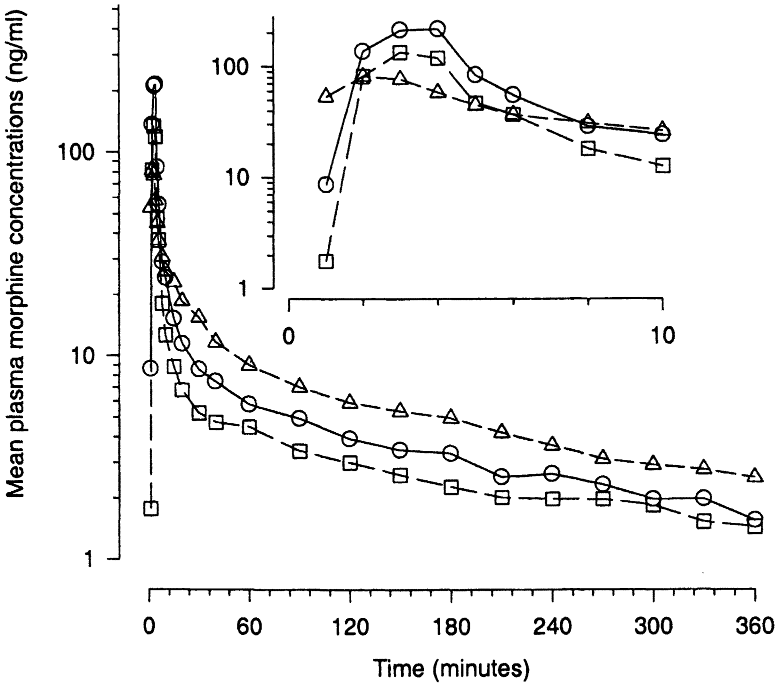

Figure 5: Average plasma morphine concentrations after 2 mg IV, 4 mg IV and 4.4 mg AERx doses of morphine sulfate. Inset: first 10 min after administration. ○ 4 mg IV, □ 2 mg IV, Δ 4.4 mg pulmonary. (Reproduced with permission from reference 4. Copyright 1997, Harcourt.)

Protein Drugs

Insulin, an example of a peptide drug, has been shown to be safe and effective when administered by the AERx system in numerous short-term clinical trials (*6-8*). The results indicate that the AERx system has equal reproducibility and efficacy when compared to subcutaneous (SC) injection. In a study with Type 1 diabetic patients, fifteen subjects were dosed four separate times with insulin, twice subcutaneously and twice using the AERx System (*8*). The loaded doses, 45 U for AERx and 8 U for injection, were calculated to yield equivalent areas under the curve for serum insulin between inhalation and injection. The injected insulin was given 30 minutes prior to a standard meal, while the inhaled insulin was given immediately prior to a standard meal. The pharmacodynamic profiles (Table III) were monitored for 10 hours after the meal.
control by either insulin injection or inhalation. By enabling dosing immediately prior to a meal, pulmonary delivery of insulin allows the patient the freedom to eat when they wish, without having to plan their insulin dose in advance of their meal.

The AERx system has been designed with the ability to deliver the desired amount of insulin, as specified by the patient, with high resolution from a single strength dosage form (*19*). A clinical trial has demonstrated a linear dose response in PK and PD profiles (*7*). Sixteen Type 1 diabetes patients received four inhaled doses of insulin on four separate days. Their blood insulin and glucose levels were monitored for 10 hours after dosing. The data from this study are shown in Figures 6 and 7, plotted as area under the insulin or glucose curve, respectively, for 10 hours after dosing as a function of dose level.

Table III: Serum Glucose Concentration Upon Repeated Dosing

	60 min. (mg/dL)		*120 min. (mg/dL)*		*300 min. (mg/dL)*	
	Mean	*SD*	***Mean***	*SD*	***Mean***	*SD*
AERx	**82**	43	**79**	45	**-11**	62
AERx Replicate	**94**	35	**85**	60	**-30**	62
SC	**89**	35	**82**	44	**-25**	58
SC Replicate	**80**	50	**85**	53	**-21**	81

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There was no statistical difference in the variability in serum glucose levels, relative to baseline, across all four doses, indicating equivalent serum glucose

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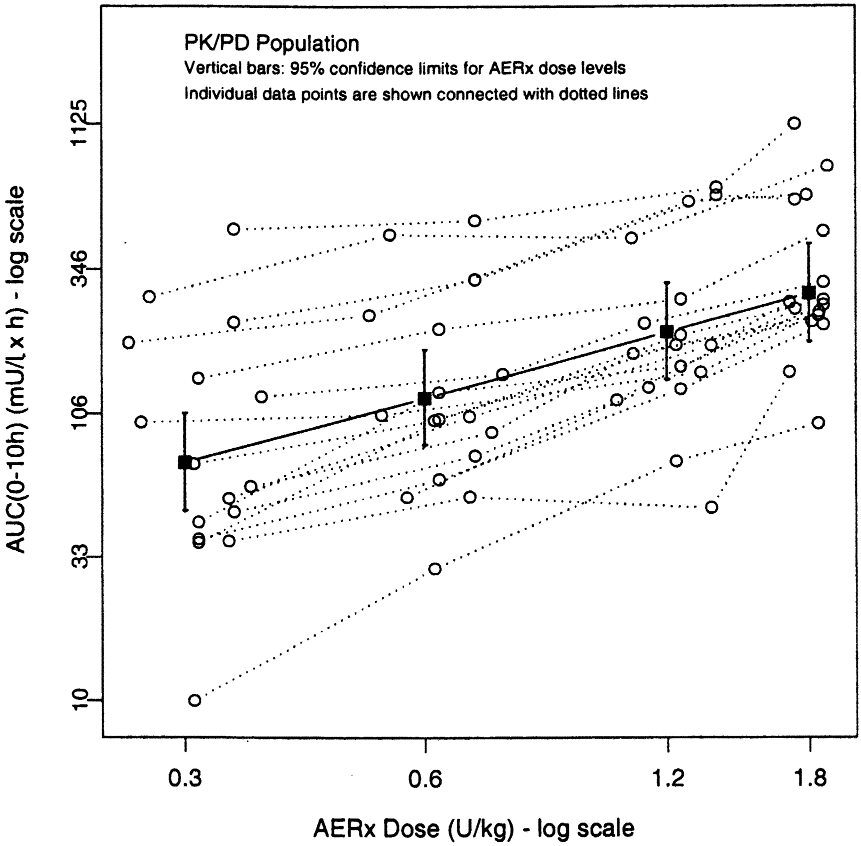

Figure 6: Dose Response of Serum Insulin Concentration, 0-10 hours following administration by the AERx system, AUC = Area Under the Curve. (Reproduced with permission from reference 7. Copyright 2000, Springer.)

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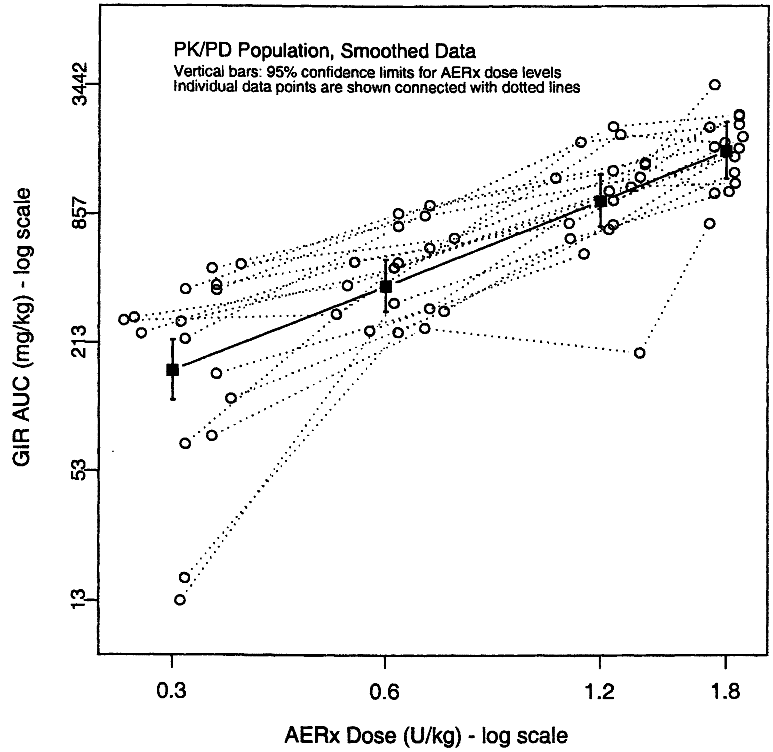

Figure 7: Dose Response of Serum Glucose Concentration, 0-10 hours following administration by the AERx system. (Reproduced with permission from reference 7. Copyright 2000, Springer.)

The estimated intra-subject variability for dose response to inhaled insulin is approximately 25.8 % (based on 0.6 and 1.2 U/kg inhaled doses). This intra-patient variability is similar to subcutaneous injection.

In these, and other studies (*15,20*), the AERx system has demonstrated the ability to maintain the integrity and activity of the protein drugs being aerosolized by the efficacy of the protein. Additionally, the AERx system aerosolization process has been shown to maintain the integrity and activity of the protein through extensive in vitro characterization (*20*).

Pulmonary Delivery for Locally Active Drugs

High-Dose Delivery

In addition to systemic delivery, the AERx system is ideal for delivery of locally active drugs as well. By changing the aerosol particle size, specific regions of the airways can be targeted. The AERx system has been used to deliver both small molecules (*17*), and protein drugs (*20*) topically to the lung. Topical delivery can require the administration of high doses. Such is the case for INS365, a small molecule nucleotide analog of uridine triphosphate, which enhances mucociliary clearance in the respiratory tract. Clinical doses of 8 mg of INS365 have been delivered to the lungs of human volunteers in only two inhalations (*17*). INS365 is stable in an aqueous formulation at 250 mg/mL for at least 1 year, and has been proven to be safe in clinical studies (*17,21*). A single dose, open label, 3-way cross over gamma scintigraphy clinical study compared the administration of INS365 using two jet nebulizers, and AERx (*17*). A comparison of the loaded dose and the delivered dose to lung for each device is shown in Table IV.

The results indicate that even when delivering highly concentrated solutions, the AERx System is much more efficient than the nebulizer, and was able to deliver an equivalent or higher dose to lung with a 90% reduction in delivery time.

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Table IV: Comparison of INS365 Delivery [a] via Jet Nebulizer and AERx

	Pari LC Plus	*Pari LC STAR*	*AERx System*
Inhalation Method	Tidal	Tidal	Bolus
Delivery Time (min)	15	15	1
INS365 Conc. (mg/mL)	10	10	250
Loaded Dose (mg)	40	40	21
Dose to Lung (mg)	6.7	7.1	8.2

[a] Evaluated in a gamma scintigraphic study in healthy volunteers.

Conclusions

Pulmonary drug delivery has a long history for drugs with a large therapeutic window, administered topically to the lungs. More recently,

In Advances in Controlled Drug Delivery; Dinh, S., et al.;
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advances have been made in delivery systems to improve the efficiency and reproducibility of delivery, making systemic drug delivery by inhalation a possibility. Aradigm's AERx system has shown the capability of delivering both protein drugs and small molecule drugs either systemically or topically. The AERx system's aerosolization process has been shown to maintain the integrity of protein drugs. It can also deliver high doses of drugs. Through built-in systems to ensure patient compliance, as well as precise control of dose, particle size distribution, inhalation flow rate and inhaled volume, the AERx pulmonary delivery system has made systemic delivery a reality.

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Chapter 6

Controlled Growth Factor Delivery for Tissue Engineering

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Controlled release of growth factors from polymer scaffolds has been an attractive platform to regenerate tissues or organs in many tissue engineering applications. In this article, we describe potential growth factors and polymers that can be adopted in tissue engineering approaches. In addition, dynamically controlled growth factor delivery, which has not been systematically exploited yet, is also depicted to envision the future perspective of growth factor delivery in tissue engineering as well as in cell transplantation. Development of polymer scaffolds that release growth factors in response to mechanical stimulation could provide a novel means to guide tissue formation in vivo.

Tissue Engineering Approach

An exciting and revolutionary strategy to treat patients who need a new tissue or whole organ is the engineering of man-made tissues or organs (*1*). Tissues can be potentially engineered using a combination of a patient's own cells combined with polymer scaffolds. In brief, tissue-specific cells are isolated

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from the patient and harvested in vitro. The cells are subsequently incorporated into three-dimensionally structured polymer scaffolds, which act as analogues to extracellular matrices found in tissues. These cell/polymer constructs can be delivered to the desired site in the patient's body, either by a minimally invasive delivery approach, or by surgical implantation following an incision (Figure 1) (*2*). In this approach, polymer scaffolds provide a space for new tissue formation, and potentially control the structure and function of the engineered tissue (*3*). A variety of tissues are being engineered using this approach including artery, bladder, skin, nerve, cartilage, bone, ligament, and tendon. Several of these tissues are now at or near clinical uses (*4*).

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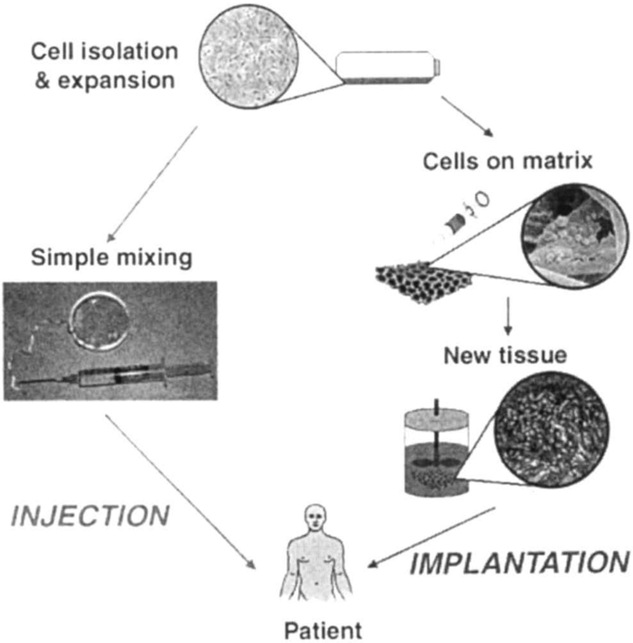

Figure 1. Schematic illustration of typical tissue engineering approaches (Reproduced from reference 2. 2001 Copyright American Chemical Society).

Growth Factor Delivery for Tissue Engineering

Growth factor delivery has been attractive to promote regeneration of certain tissues, including blood vessels, cartilage, and bone (*5*). There has been a significant need for controlled growth factor delivery using polymer matrices, as this approach offers numerous advantages over the conventional means of protein delivery, which include intravenous bolus injection and tissue injection. When a growth factor is administered via bolus injection, its concentration is within the therapeutic window for only a short time period due to the inherent instability of growth factors, and the administration often must be repeated. In contrast, controlled delivery systems can potentially maintain the concentration of growth factors within the therapeutic window for prolonged time periods, which may enable one to decrease the number of dosages, and to achieve efficient drug administration (*6*).

Potential Growth Factors

A number of growth factors, including bFGF, VEGF, PDGF, BMP, NGF, and EGF have been identified to play important roles in tissue regeneration. The major angiogenic growth factors, extensively studied to date, are bFGF and VEGF. Basic fibroblast growth factor (bFGF) belongs to one of 22 members of FGF family, and the ability of bFGF to induce angiogenesis in animals has been demonstrated (Figure 2) (*7*). The vascular endothelial growth factor (VEGF) family currently includes 6 related proteins, and VEGF-A comes in at least five isoforms including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. $VEGF_{165}$ is the most common and biologically active form, and acts as a specific mitogen to endothelial cells (*8*). However, single intravenous injection of VEGF and bFGF was not effective to enhance angiogenesis in animals, as protein growth factors are inherently instable in the blood stream. For example, the half-life of VEGF is 90 min following systemic administration (*9*). Its short half-life is related to proteolytic degradation in the circulation, and binding of these heparin-binding factors to large quantities of heparan sulfate in the lungs (*10*).

Bone morphogenetic proteins (BMPs) are classified within the family of transforming growth factor-β (TGF-β). BMPs are prominent molecules necessary for development of tissues and organs early in embryogenesis, and are also involved in stimulating bone growth and morphologic differentiation (*11*). BMPs are sequestered primarily in bone and are liberated following bone damage. They act by promoting differentiation of pluripotent cells into osteogenic cells. BMPs have been used to regenerate bony tissues in animals, and recently shown great potential in clinical trials (*12*). Nerve growth factor (NGF) promotes the survival and neurite outgrowth of degenerating cholinergic neurons, found in many disorders of the central nerve systems such as Alzheimer's disease, Parkinson's disease, and Huntington's chorea (*13*). In addition, growth factors from the neutrotrophin family including β-NGF, brain-derived neutrophic factor (BDNF), and neutrophin-3 (NT-3) have potential to enhance peripheral nerve regeneration (*14*). Epidermal growth factor (EGF) is a small (6100 Da) growth factor that is heat stable and resistant to proteolytic degradation (*15*). Wide ranges of cell types, which play an important role in wound healing and tissue regeneration, express the EGF receptor. Continuous

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administration of EGF into animals has been shown to increase proliferation of neural precursor cells, suggesting a potential use for this factor in treatment of neural disorders (*16*).

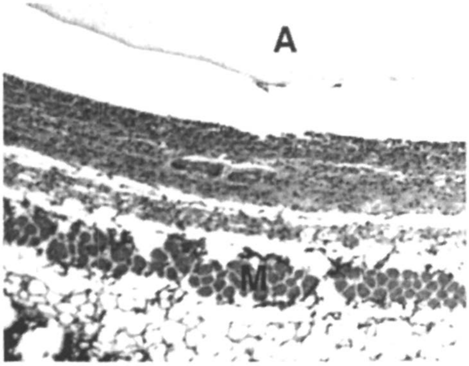

Figure 2. Photomicrograph of tissue section following subcutaneous implantation of alginate hydrogels containing bFGF into SCID mice. Tissue sections were taken after two weeks, and stained with hematoxylin and eosin. Enhanced angiogenesis was observed between alginate hydrogel (A) and muscle layer (M).

Potential Polymers

Extracellular matrices (ECMs), comprised of various amino acids and sugar-based macromolecules, are known to bring cells together, control tissue structure, regulate the function of the cells, and allow the diffusion of nutrients, metabolites, and soluble factors including growth factors to and from cells. Polymers can potentially mimic many roles of ECMs found in tissues. Various types of polymers have been utilized to date in tissue engineering applications. Many synthetic polymers, such as aliphatic polyesters, polyanhydrides, poly(ortho ester)s, and polypeptides have been considered potential implantable materials. These polymers are typically used in the physical form of foams, sponges, and films. This type of polymer scaffold requires the surgeon to make incisions sufficiently large to enable placement of the polymer/cell constructs. In contrast, natural polymers, such as alginate, agarose, chitosan, hyaluronate, fibrin, and collagen, can be used to form hydrogels or micro beads, and used as an injectable material. The use of injectable materials enables the clinician to transplant the polymer/cell construct in a minimally invasive manner. Certain polymers such as polyanhydrides and collagen can be used in both forms (*17*).

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Polymers for Implantable Materials

Aliphatic polyesters including poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and copolymers of these materials (PLGA) are the most widely used synthetic biodegradable polymers in medicine. These polymers have a long history of medical applications, and are considered safe in many clinical uses by the FDA. PLA has better solubility in organic solvents than PGA due to the methyl group in PLA. However, PLA is less labile to hydrolysis due to steric hindrance of the methyl group, resulting in slower degradation. PLGA can be readily synthesized, and their physical properties and degradation rate are controlled by the ratio of glycolic acid to lactic acid as well as the molecular weight. Promising processing techniques to generate highly porous polymer scaffolds for tissue engineering applications include gas foaming/particulate leaching, phase separation, emulsion freeze-drying, and fiber extrusion and fabric formation (*18*).

A number of other synthetic polymers can be also used to engineer tissues (*19*). Polycaprolactone is one of aliphatic polyesters with high solubility in organic solvents and low melting temperature. However, its degradation rate is much slower than PGA and PLA (*20*). Polyanhydrides are usually copolymers of aromatic diacids and aliphatic diacids, and degrade by surface erosion (*21*). Poly(ortho ester)s degrade by gradual surface erosion, and have been known as useful materials for controlled growth factor delivery (*22*). The synthesis of polypeptides to mimic natural proteins has been exploited, as they are major components of natural ECMs. Recently, new strategies to synthesize polypeptides with well-defined structures and a wide range of molecular weights were reported, which could precisely control the sequence of amino acids and enhance the solubility of polypeptides in common organic solvents (*23*).

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Polymers for Injectable Materials

A number of natural polymers have been utilized in an injectable form for tissue engineering applications (*2*). Alginate is one potential polymer for this purpose due to its simple gelation with divalent cation (e.g., Ca^{2+}), as well as its biocompatibility, hydrophilicity, and relatively low cost. Alginate can be used in an injectable form by either being preformed into small beads, or by simple injection after gelation (*19*). Hyaluronate is one of glycosaminoglycan components of natural ECMs, and its enzymatic degradation occurs by hyaluronidase. Hyaluronate has been used in many biomedical applications. However, it has poor physical strength and this has limited its applications. Chitosan has been known to be biocompatible, biodegradable, and of low toxicity. Chitosan has been used as a cell substrate to regenerate tissues (*24*). Fibrin gels can be produced from the patient's own blood, and have also been utilized to engineer many tissues (*25*). However, fibrin gels also have limitations in mechanical strength, and this prevents their use in certain applications. Collagen is the best-known tissue-derived natural polymer. It is the main component of many mammalian tissues including skin, bone, cartilage, tendon, and ligament. Collagen has been used as a tissue culture scaffold or artificial skin due to the easy attachment of many different cell types, regardless of its limited range of physical properties and high cost (*26*). Covalent cross-linking of hyaluronate and collagen has been widely investigated as a means to broaden the range of mechanical properties available from these materials (*27*).

Dynamically Controlled Growth Factor Delivery

ECMs of natural tissues are regarded as depots of various growth factors, which are released to cells to affect a variety of physiologic processes. Synthetic and naturally derived polymers can be similarly used as depots and delivery vehicles of protein growth factors. However, most tissues in the body are subjected to mechanical stimuli, and this signaling should be considered in the design of polymer scaffolds that release growth factors. We have found that delivery systems of protein growth factors can be designed to respond to compressive stimulation, and this response can be used to regulate the local concentration of the growth factors in vivo (*28*).

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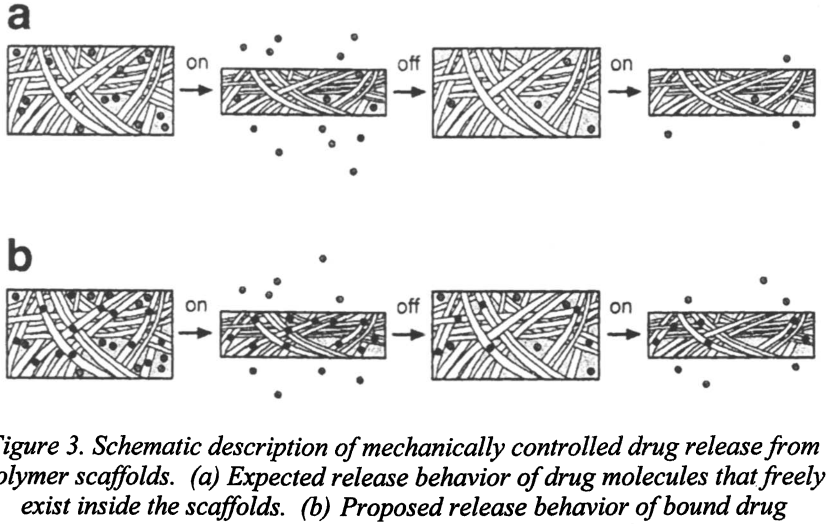

Figure 3. Schematic description of mechanically controlled drug release from polymer scaffolds. (a) Expected release behavior of drug molecules that freely exist inside the scaffolds. (b) Proposed release behavior of bound drug molecules that interact with the scaffolds. (○, free drug; ●, bound drug).

A potentially critical feature to design this type of delivery systems is the reversible binding of protein growth factors to the polymer scaffolds. Two different types of model systems can be suggested depending on the interaction of drug with a polymer (*29*). One model system is filled with drug molecules that do not bind or interact with polymer scaffolds. The release of these free drug molecules can be enhanced by simple compression to the scaffold due to the increased pressure within the matrix (Figure 3a). However, mechanical signaling does not significantly contribute to the release behavior of this type of drug molecules, due to the depletion of free drug molecules even during the relaxation time period. The other model system is composed of both free and bound drug molecules, which interact with the polymer through ionic or secondary binding forces. When this type of system is subjected to repeated compressional loading, free drug molecules can be released in response to the signals, and subsequently replenished during relaxation by dissociation of previously bound drug. This allows the system to respond to repeated cycles of mechanical stimulation (Figure 3b).

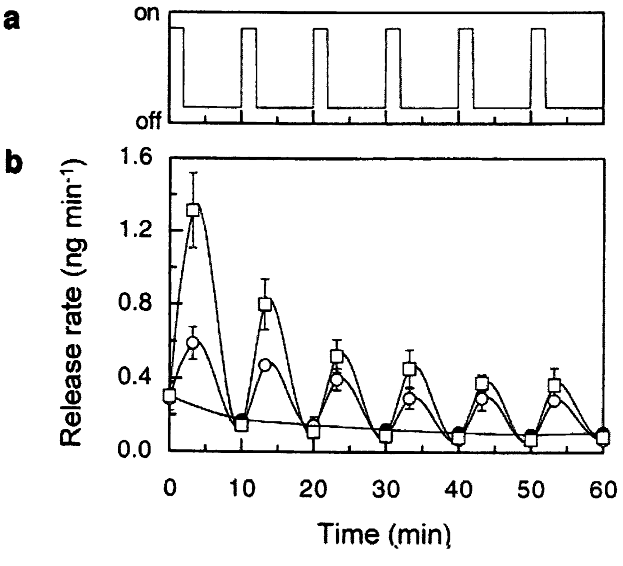

Figure 4. (a) Diagram of mechanical stimulation, which involved six cycles of compression for 2 min followed by relaxation for 8 min. (b) Release rate of VEGF from alginate hydrogels under 10% (○), 25% (□) strain amplitude, and no compression as a control (●)(Reproduced from reference 28. 2000 Copyright Macmillan Magazines Ltd.).

To confirm the importance of the drug-polymer interaction, alginate gels incorporating vascular endothelial growth factor (VEGF) were subjected to compressional loading, and the release of VEGF was monitored. VEGF was chosen as a model growth factor due to its use in many clinical applications, and

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its reversible binding interaction with polysaccharides (*30*). In the absence of mechanical stimulation, the release rate was fairly constant. However, the release rate under mechanical stimulation increased up to 5 times higher than that of control gels without stimulation, and this increase can be regulated by the strain amplitude of the compression (Figure 4). In this situation, free VEGF would be released following each incident of stimulation, but the system would re-equilibrate during the subsequent relaxation by the disassociation of bound VEGF from the gel. The cumulative release profile showed a stepwise increment with mechanical stimulation, and the total amount of VEGF released from the hydrogels was up to 2 times more than that of control conditions (*28*).

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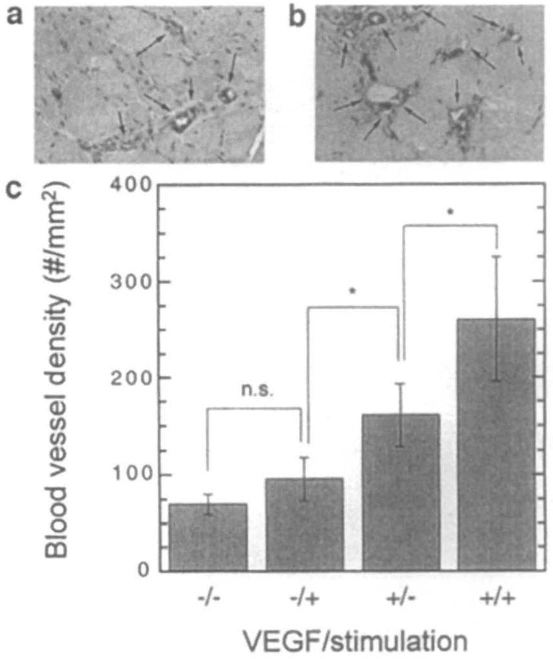

*Figure 5. Photomicrographs of representative tissue sections surrounding (a) VEGF-incorporated gel/no mechanical stimulation (+/-) and (b) VEGF-incorporated gel/mechanical stimulation (+/+). (c) Blood vessel density at each experimental condition. Gels without VEGF under static conditions (-/-) and under mechanical stimulation (-/+) were also implanted as a control. Arrows indicate CD31-stained blood vessels. n.s. indicates no statistical difference. *statistical significance at a level of $p < 0.05$ (Reproduced from reference 28. 2000 Copyright Macmillan Magazines Ltd.).*

The ability of mechanical stimulation to upregulate growth factor release in vivo was next tested. VEGF was also used as a model factor as it is a potent endothelial cell-specific mitogen. Alginate hydrogels containing VEGF were implanted into nonobese diabetic (NOD) mice following femoral artery ligation and mechanical stimulation was applied to the implantation site to determine if increased collateral circulation could be achieved (*28*). Enhanced blood vessel formation after 14 days was observed in mechanically stimulated implant sites (Figure 5). The density of blood vessels increased 1.6 times more than that without mechanical stimulation ($p < 0.05$). Hydrogels without VEGF were also used as a control, and no significant blood vessel formation was observed either with or without mechanical stimulation in this situation.

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Conclusions

There are various approaches that could be used to regulate the growth factor release from polymer scaffolds, including control of both physical and biological performance parameters of the scaffolds. In addition, external stimuli (e.g., temperature, pH, ultrasound, electric or magnetic fields, and mechanically dynamic environment) should also be considered in the design of delivery systems for growth factors (*31*). One important, but previously unexploited, external stimulation in the body is mechanical signals. We have demonstrated that these inputs can actively trigger the release of interactive drugs (i.e., growth factors) from polymer scaffolds in a controlled manner. The findings of this study may have a number of potential applications for tissue engineering and drug delivery.

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Chapter 7

Controlled Drug Delivery to Drug-Sensitive and Multidrug Resistant Cells: Effects of Pluronic Micelles and Ultrasound

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We are developing a new modality of drug targeting to tumors, based on drug encapsulation in polymeric micelles followed by localized release at the tumor site triggered by focused ultrasound. In the present paper, effects of micelles and ultrasound on the intracellular uptake and cytotoxicity of anthracycline drugs in drug-sensitive and multidrug resistant (MDR) cells are discussed. Significant sensitization of MDR cells by Pluronic micelles and ultrasound was observed, presumably resulting from the suppression of the intracellular energy metabolism and the permeabilization of the cytoplasmic acidic vesicles, which enhanced drug uptake and allowed drug trafficking into cell nuclei. Both effects were enhanced by ultrasound.

Introduction

The technique of drug targeting to tumors that we are developing involves drug encapsulation in polymeric micelles followed by controlled release at the target site triggered by focused ultrasound (*1-6*). These polymeric micelles are formed by hydrophobic-hydrophilic block copolymers (*1-14*). Their amphiphilic character, size (~10 - 30 nm) and surface properties provide for a high drug loading capacity and long circulation time in the vascular system, which makes them attractive drug carriers (*7, 8*). The important advantage of polymeric micelles is a so-called enhanced penetration and retention effect that provides for a selective accumulation of micellar-encapsulated drugs in solid tumors (*15-17*). We have shown that when drugs were encapsulated in polymeric micelles, their intracellular uptake was substantially reduced; these drug shielding properties of micelles are advantageous for preventing unwanted interactions with healthy tissues (*1-6*). The challenge to be met, however, is to ensure drug uptake by tumor cells. In the current work, we have been experimenting with ultrasound to activate intracellular drug uptake, with a considerable success. We have found that ultrasound induced drug release from micelles in a spatially and temporally controlled manner and enhanced the intracellular uptake of a free and micellar-encapsulated drug; in addition, micellar delivery combined with ultrasound dramatically sensitized multidrug resistant (MDR) cells to the action of drugs.

Ultrasound consists of pressure waves with frequencies at or greater than 20 kHz generated by piezoelectric transducers that change an applied voltage waveform into mechanical translation of the face of the transducer. Like optical or audio waves, ultrasonic waves can be focused, reflected, refracted, and propagated through a medium, thus allowing the waves to be directed to and/or focused on a particular volume of tissue. The technology for ultrasonic wave control and delivery are well advanced and pervasive in the areas of biomedical imaging and flow measurement. Ultrasound is also used in hyperthermic cancer therapy. The main advantage of ultrasound is its non-invasive nature: the transducer is placed in contact with a water-based gel or water layer on the skin, and no insertion or surgery is required. Ultrasound technology allows for a high degree of spatial and dynamic control due to a favorable range of energy penetration characteristics in soft tissue and the ability to shape energy deposition patterns.

The polymeric micelles of this study were formed by a Pluronic P-105 copolymer, which is a tri-block ABA-type copolymer of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO); Pluronic copolymers have attracted special attention as modulators of a biological response due to the combination

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of advantageous properties that culminated in the hypersensitization of drug resistant cells reported by Kabanov and Alakhov *(9,10,12)*.

Pluronic copolymers in a *micellar* form have been evaluated as drug carriers by our research team *(1-6,18-20)*. The fundamental difference between our approach and that of Kabanov *et al.* is that we take advantage of the *drug shielding* and *targeting* properties of Pluronic *micelles*, while the technique used by Kabanov *et al.* relies on the hypersensitization properties of Pluronic *unimers (9,10,12)*.

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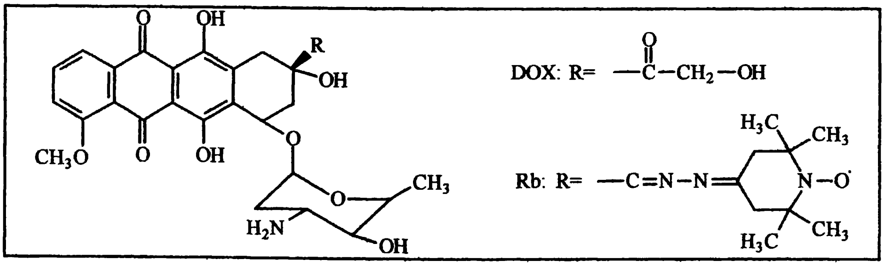
Materials and Methods

Anthracycline Drugs

Two anticancer anthracycline drugs have been studied: doxorubicin (DOX) and Ruboxyl (Rb). DOX is an intercalating drug that stacks between paired bases in DNA. DOX was purchased from Sigma (St. Louis, MO). Rb is a paramagnetic analog of DOX synthesized by conjugating a paramagnetic nitroxyl radical to DOX (US Patent #4,332,934; 1982) (Scheme 1). Rb was kindly provided by Dr. A. Shapiro (Russian Academy of Sciences). The paramagnetic label of Rb molecule can report whether the drug is free, encapsulated, internalized by the cell, or bound to protein carrier in blood.



Scheme 1. Molecular structures of DOX and Rb

Cell lines

Promyelocytic leukemia HL-60 cells grown in suspensions and ovarian carcinoma A2780 drug-sensitive and MDR cells grown in adherent monolayers were used in this study; the MDR cell line A2780/ADR was kindly provided by Dr. T.C. Hamilton (Fox Chase Cancer Center, PA). Cells were cultured in a complete RPMI 1640 medium, which in case of A2780/ADR cells included 0.8 µg/ml DOX for maintaining resistance.

Drug Introduction. Measuring Intracellular Drug Uptake

The cells in suspensions (for HL-60) or monolayers (for A2780 and A2780/ADR) were incubated or sonicated for various time periods with various concentrations of DOX or Rb in either RPMI 1640 or in the medium comprising various concentrations of Pluronic P-105; the solutions were sterilized by filtration through a 0.2 µm filter. Upon the completion of incubation, cells were fixed with a 3% formalin, washed with cold PBS, tripsinized and resuspended in PBS.

Three independent techniques were used to measure the intracellular drug uptake: depletion from the incubation medium, accumulation in the cells (in cell lysates), and flow cytometry. Upon the completion of the incubation, cells were fixed, tripsinized, separated from the incubation medium and washed as described above in the drug introduction section; then the cells were counted with a hemacytometer and divided into two parts; in one part, drug uptake was measured by flow cytometry; the other part of the cells was lysed by incubating with a 2% sodium dodecyl sulfate (SDS). Drug fluorescence in cell lysates was measured using photon-counting spectrofluorometer (ISS, model PC-1, Champaign, Il); the excitation wavelength was 488 nm and the emission wavelength was 590 nm. The mean of five measurements was used for the calculations. Calibration experiments showed a linear dependence of DOX or Rb fluorescence intensity on drug concentration in PBS, Pluronic, or SDS at the concentration range used. The amount of the internalized drug was normalized to the cells' concentration in lysates characterized according to a protein content measured by the BCA assay. Fluorescence histograms were recorded with a Facscan (Beckton Dickinson) flow cytometer and analyzed using CellQuest software supplied by the manufacturer. A minimum of 10,000 events was analysed to generate each histogram.

Sonication

Sonication of ovarian carcinoma cells grown in 6-well or 96-well plates was performed at 37^{0}C in a sonication bath operating at 69 kHz at power densities ranging from 1.66 W/cm^2 to 10.66 W/cm^2 (Sonicor, Copiaque, NY).

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In our experiments, no detachment of cells from the substrate during ultrasonic exposure was observed. Sonication of HL-60 cells at 20 kHz was performed in test tubes inserted into cup horn bath (Sonics and Materials, Newton, CT).

Assessing the effect of micellar delivery and sonication on drug cytotoxicity

Cells were seeded into the 96-well plate at a number of $2x10^4$ cell/well. They were cultured overnight to allow attachment. After that, the culture medium was replaced with a drug- and Pluronic-containing medium and cells were incubated or sonicated; sonication was performed at 69 kHz or 20 kHz for 10 to 30 min; overall cell incubation time with the drug was 3 h. Upon the completion of incubation, the drug-containing medium was removed, cells were washed three times with cold PBS, and cultured in a drug-free medium for 72 hours. Cell viability was assessed by the MTT assay *(21)*.

EPR spin trapping study

Nitron spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Sigma. DMPO solution was added to the sample to produce a final concentration of 0.05 M. The DMPO radical trap converted the short-lived hydroxyl radicals into longer-lived nitroxide radicals, which could be observed by the conventional EPR technique.

Fluorescence microscopy

Cells were fixed with a 3% formalin, sealed on glass slides and visualized at 100X magnification by fluorescence microscopy with 527-552 nm excitation and 577-632 nm emission wavelength (Eclipse E800, Nikon, Tokyo, Japan).

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Results and Discussion

Ultrasound-Induced Drug Release from Micelles

Under the action of ultrasound, drug was partly released from Pluronic micelles *(6)*. Drug release measurements were based on the decrease of the fluorescence intensity when drug was transferred from the hydrophobic environment of the micelle core into the aqueous environment. Drug release from micelles was observed for various types of micelles with the "soft" (above glass transition) or "solid" (below glass transition) cores. The first type was represented by Pluronic micelles; the second by diblock copolymers of poly(ethylene oxide) (PEG) with either poly(β-benzyl aspartate) (PBBA) or poly(D,L-lactate) (PDLLA). An example of a DOX release profile from the MeO-PEG/PDLLA-Metocryloyl diblock copolymer under continuous wave (CW) and pulsed ultrasound is shown in Figure 1. This Figure also shows that the released drug was quickly re-encapsulated at the "ultrasound off" phase, which suggested that upon leaving the sonicated volume, the non-internalized drug would circulate in the encapsulated form, thus preventing unwanted drug interactions with normal tissues.

Factors that affected the degree of drug release included: (1) ultrasound frequency (*higher* release at *lower* frequencies); (2) power density (higher release at higher power densities); (3) ultrasound pulse duration (increased drug release with increasing pulse duration up to 0.5 s, followed by a constant release at pulse durations longer than 0.5 s); (4) drug/micelle interaction (lower release of a more hydrophobic drug that is deeper inserted into the micelle core); (5) micelle concentration (higher drug release at lower polymer concentrations [all concentrations being above the CMC], presumably due to a higher local concentration of drug in the micelle core).

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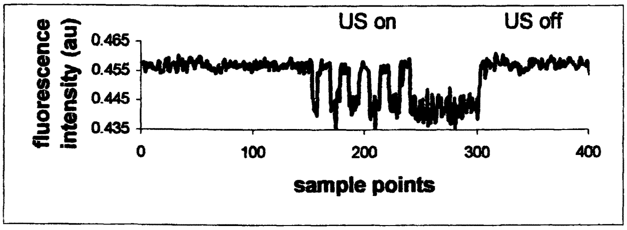

Figure 1. DOX release from MeO-PEG/PDLLA-Metocryloyl diblock copolymer micelles under CW and pulsed 67-kHz ultrasound; power density 10.4 W/cm^2.

Ultrasound frequency is an important and easily controllable parameter. We explored drug release as a function of ultrasound frequency in the range of 20 kHz to 3 MHz. Drug release from micelles was highest at 20 kHz and dropped with increasing ultrasound frequency *(6)*. For instance, a 10% release of the encapsulated DOX from a 10% Pluronic micellar solution proceeded at a power density of 0.058 W/cm^2 under 20-kHz ultrasound, at a power density of 2.8 W/cm^2 under 67-kHz ultrasound, and at a power density of 12 W/cm^2 under 1.0-MHz ultrasound.

The dependence of the degree of drug release on ultrasound power density projected into the coordinate origin suggesting that a process other than transient cavitation was responsible for drug release (Figure 2). In contrast, the dependence of the degree of cell sonolysis on power density demonstrated a distinct threshold nature suggesting the involvement of transient cavitation in that process (Figure 2); the same was also implied by a very good correlation between the degree of cell sonolysis and the concentration of trapped hydroxyl radicals formed upon cavitation collapse. Different dependence of drug release and cell sonolysis on power density provided a window of power densities, inside which a noticeable drug release from micelles was not accompanied by a substantial cell lysis (Figure 2). This is important for the clinical application of the above technique. Extensive cell lysis should be avoided as it can cause inflammation of surrounding tissues.

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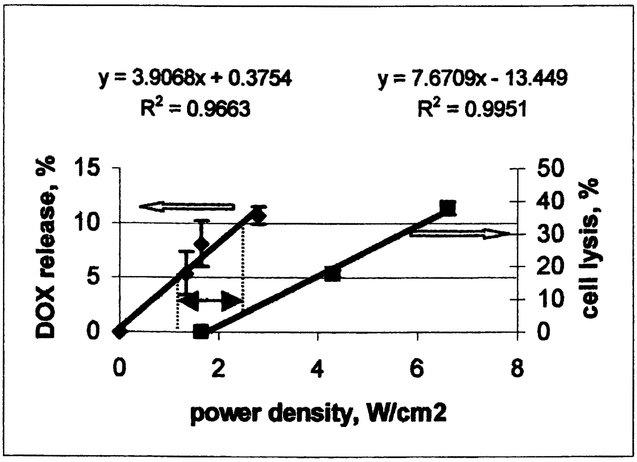

Figure 2. Effect of power density on the ultrasound-induced drug release from a 10% Pluronic P-105 micelles and on HL-60 cell sonolysis; ultrasound frequency 67 kHz, DOX concentration 10 μg/ml.

Intracellular Drug Uptake by Drug-Sensitive and MDR cells: Effect of Ultrasound

Because free drug is internalized much more effectively than when encapsulated, ultrasound-induced drug release from micelles enhances drug uptake, which is an important component of the mechanism of the ultrasonic enhancement of micellar drug delivery. In addition, ultrasound enhances the internalization of the free drug and micellar encapsulated drug, mostly due to the cell membrane sonoporation and other ultrasound-induced cellular changes that enhance the accessibility of various cellular structures to drugs and micelles *(19,20)*.

The effect of continuous wave (CW) 67-kHz ultrasound on DOX uptake by drug-sensitive A2780 and MDR A2780/ADR ovarian carcinoma cells is shown in Tables 1 and 2. The data indicated that ultrasound enhanced drug uptake by both drug-sensitive and MDR cells. The combined action of Pluronic micelles and ultrasound substantially decreased (Table 1) or even eliminated (Table 2) the differences between drug-sensitive and MDR cells; moreover, drug uptake by the MDR cells from/with Pluronic micelles was close to that by drug-sensitive cells from a conventional medium without ultrasound (Table 2). Increased uptake was accompanied by a dramatic sensitization of the MDR cells to the action of DOX (Figure 3). Note that in a conventional medium, about 55% of the MDR cells were highly resistant to the action of DOX even at a concentration of 50 μg/ml (Figure 3); the fraction of highly resistant cells decreased to about 30% upon a 10-min sonication by a 67-kHz ultrasound; the highly resistant fraction of the cells was completely eliminated in the presence of Pluronic micelles; all MDR cells were killed under a combined action of Pluronic micelle and ultrasound at a DOX concentration as low as 0.78 μg/ml.

Intracellular Drug Uptake by Drug-Sensitive and MDR cells: Effect of Pluronic Unimers and Micelles

Note that when DOX was delivered in Pluronic micelles, *its uptake by drug-sensitive cells dropped while the uptake by the MDR cells increased* (Table 1). The decrease of drug uptake by sensitive cells resulted from the shielding effect of micelles. The increase of DOX uptake by the MDR cells in the presence of Pluronic micelles was presumably caused by a Pluronic-induced deactivation of drug efflux pumps in MDR cells, as was previously reported for Pluronic unimers by Kabanov's group *(10 – 12)*. In the MDR cells, deactivation of efflux pumps prevailed over the shielding effect of Pluronic micelles that was clearly expressed in drug-sensitive cells. The increase of DOX uptake by the

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MDR cells was more pronounced at a Pluronic concentration of 0.1% than at 10% (Table 1). This is because at a concentration of 0.1%, no micelles were formed (the CMC for the formation of dense micelles for Pluronic P-105 at 37°C being between 0.1 wt% and 1 wt%), and therefore no drug shielding effect (which is opposite in sign to the deactivation of efflux pumps), was exerted. Still, drug delivery in micelles (rather than with unimers) has a great advantage of providing for drug targeting. In addition, as shown in Figure 3, delivery in micelles combined with the application of ultrasound results in a very effective sensitization of the MDR cells.

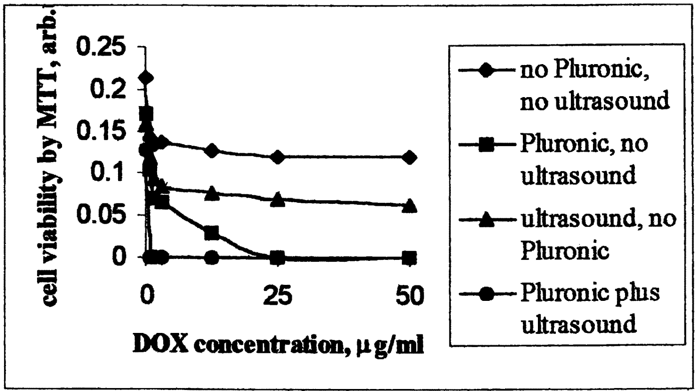

Figure 3. Effect of Pluronic micelles and ultrasound on the viability of the MDR A2780/ADR cells; cell incubation with Pluronic and DOX for 3h followed by culturing in a drug-free medium for 72 h.

Mechanism of the MDR Cell Sensitization by Pluronic Micelles

To confirm that the deactivation of efflux pumps was involved in the sensitization of the MDR cells by Pluronic micelles, we studied the effect of Pluronic on the intracellular uptake of Rhodamin 123 (Rh123) that is a substrate of the Pgp efflux pump. The results are shown in Figure 4. Incubation with progressively increasing concentrations of Pluronic *unimers* (concentration range up to 0.1 wt%) resulted in the increase of Rh 123 uptake by both drug-sensitive and MDR cells. The effect of Pluronic observed for the sensitive cells suggested that there were some working efflux pumps in these cells as well; however, the Rh 123 uptake from RPMI 1640 by sensitive cells was about a four-fold higher than that by the MDR cells suggesting that the efflux pumps were much less active in the sensitive cells (Figure 4).

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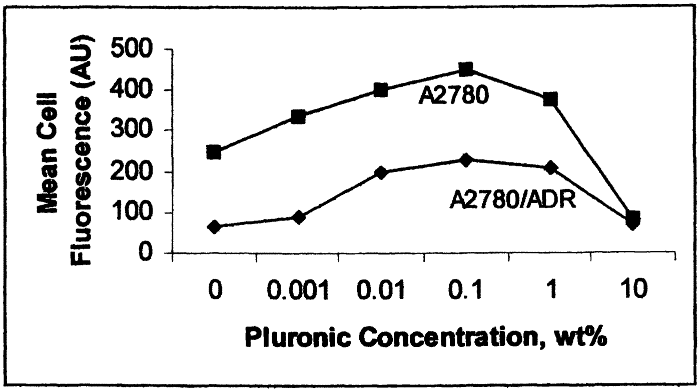

Figure 4. Effect of Pluronic P-105 on the Rh 123 uptake by drug-sensitive and MDR ovarian carcinoma cells.

The difference in the Rh 123 uptake between drug-sensitive and MDR cells dropped with increasing concentration of Pluronic unimers; the difference was only about two-fold at a Pluronic concentration of 0.1% (Figure 4), indicating that Pluronic unimers effectively deactivated efflux pumps of the MDR cells.

When Pluronic concentrations increased above the CMC, Rh 123 uptake by the drug-sensitive cells dropped due to the shielding effect of Pluronic micelles (as was shown above for DOX and Rb). For the MDR cells, the drop of the Rh 123 uptake from Pluronic micelles was much less pronounced due to the competition between the drug shielding effect of Pluronic micelles and the deactivation of the efflux pumps that act in the opposite direction.

A mechanism of the efflux pump deactivation is presumably related to the decreased energy production in the MDR cells under the action of Pluronic unimers and micelles discovered simultaneously by us *(18)* and Kabanov's group *(22)*. A decreased mitochondrial activity observed in these works may be related to the enhanced membrane fluidity in the presence of Pluronic micelles recorded by the EPR technique; our EPR results *(18)* were confirmed by others in experiments using the fluorescent membrane probe DPH *(23)*.

Effect of Pluronic Micelles on the Intracellular Drug Trafficking

Pluronic micelles not only enhanced the intracellular drug uptake but also affected drug trafficking in the MDR cells. When drugs were delivered in PBS,

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they did not penetrate into the nuclei of the MDR A2780/ADR cells, as examplified for Rb in Figure 5 A; however, when delivered in Pluronic micelles, drugs accumulated in the cell nuclei (Figure 5 B).

Table 1. Effect of a 69-kHz ultrasound on DOX uptake by drug-sensitive and MDR ovarian carcinoma cells; ultrasound power density 3.2 W/cm^2; exposure time 30 min.

Cell line	*Incubation medium*	*Mean fluorescence, arb.u.*	
		Non sonicated	*Sonicated*
A2780	RPMI 1640	229.2 ± 15.4	294.1 ± 21.8
	0.1% P-105	237.4 ± 16.0	274.7 ± 24.0
	10% P-105	124.4 ± 11.2	160.0 ± 6.1
A2780/ADR	RPMI-1640	52.3 ± 5.0	58.2 ± 5.3
	0.1% P-105	143.6 ± 15.4	191.8 ± 15.6
	10% P-105	72.8 ± 4.1	104.7 ± 5.2

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Table 2. Effect of a 69-kHz ultrasound on DOX uptake by drug-sensitive and MDR ovarian carcinoma cells; ultrasound power density 6.7 W/cm^2; exposure time 30 min.

Cell line	*Incubation medium*	*Mean fluorescence, arb.u.*	
		Non sonicated	*Sonicated*
A2780	RPMI 1640	287 ± 20.2	450 ± 25.4
	0.1% P-105	320 ± 28.0	501 ± 30.4
	10% P-105	91 ± 13.7	249 ± 20.2
Co-culture A2780 + A2780/ADR	RPMI-1640	147 ± 13.2	199 ± 16.7
	0.1% P-105	252 ± 15.1	429 ± 23.4
	10% P-105	89 ± 10.2	231 ± 12.8

To evaluate the mechanism of the Pluronic micelle effect on the intracellular drug trafficking, we studied the intracellular distribution of Pluronic molecules in drug-sensitive and MDR cells upon cell incubation with a fluorescently-labeled Pluronic unimers and micelles *(24)*. Pluronic P-105 was lebeled with a pH-dependent fluorescent label that manifested a three-fold lower fluorescence in the acidic than in the neutral environment.

Both drug-sensitive and MDR cells incubated with a fluorescently labeled Pluronic acquired fluorescence, indicating Pluronic internalization. Application of ultrasound enhanced Pluronic intracellular accumulation and changed its

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intracellular distribution; under ultrasound, Pluronic was released from the confining cytoplasmic compartments into the cytosol.

When the drug-sensitive and MDR cells were incubated with micellar Pluronic solutions (1%), flow cytometry histograms manifested bimodal distribution of cell fluorescence (Figure 6) *(24)*. For the MDR cells, the bimodal distribution of cell fluorescence was already observed at very low Pluronic concentrations in the incubation medium (below the CMC), while for the drug-sensitive cells it was observed only above the CMC (Figure 6). Cell population characterized by a higher fluorescence intensity presumably resulted from Pluronic transfer from the acidic environment of cytoplasmic vesicles (endosoms or lysosoms) into the neutral environment of the cytosole and cell nuclei, which suggested the permeabilization of the membranes of acidic vesicle by Pluronic molecules. The data suggested that the membranes of acidic vesicles of MDR cells were more susceptible to the permeabilizing action of Pluronic than those of drug-sensitive cells. This is an important finding because positively charged drugs usually end up in acidic vesicles. This is especially pronounced in the MDR cells due to a high pH gradient between the vesicles and the cytosol *(25)*. Similar effect of the permeabilization of acidic vesicles of MDR cells under the action of Pluronic L61 unimers was reported earlier by Kabanov and Alakhov *(26)*. Acidic vesicles create a second barrier on the way of anthracyclin drugs to their target localized in cell nuclei (the first barrier being a plasma membrane). We hypothesize that the permeabilization of acidic vesicles by Pluronic molecules caused drug release from the vesicles into the cytosol, followed by drug trafficking into cell nuclei (see Figure 5 A, B), which additionally sensitized the MDR cells.

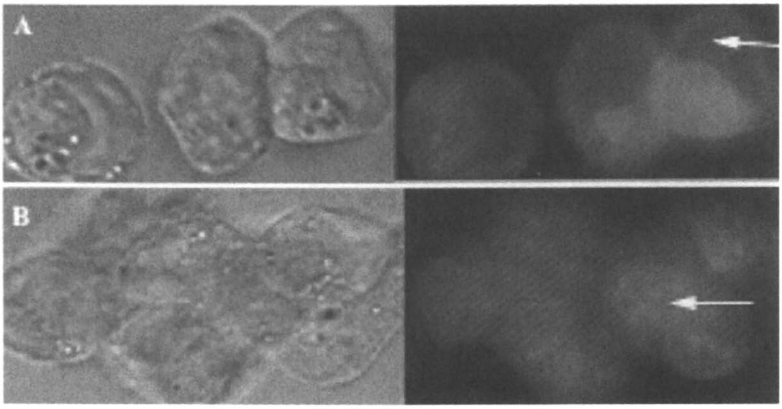

Figure 5. Rb distribution in MDR A2780/ADR cells when Rb is delivered (A) - in PBS and (B) - in a 10% solution of Pluronic P-105 in PBS; cell nuclei indicated by arrows.

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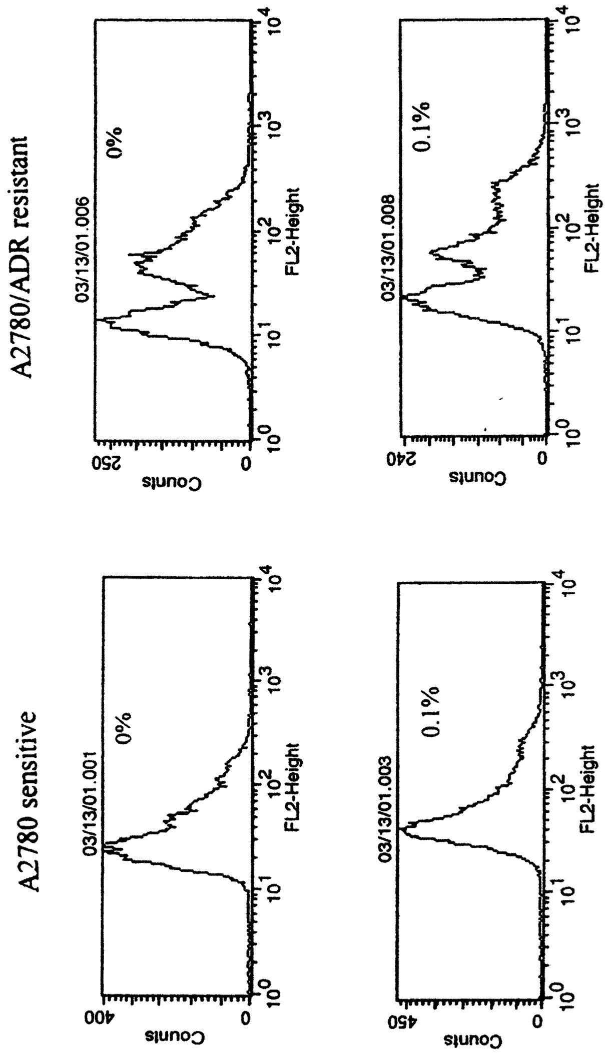

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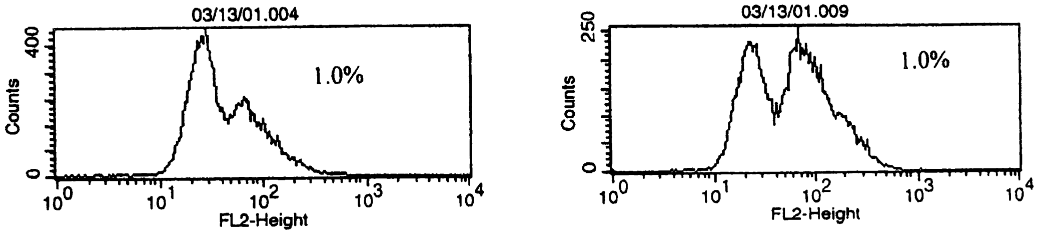

Figure 6. Fluorescence histograms of drug-sensitive (left column) and MDR (right column) cells incubated with a mixture of a fluorescently labeled (0.001 wt %) and unlabeled Pluronic P-105; the concentration of unlabeled Pluronic is indicated in the Figure. Reproduced with permission from Reference 24. Copyright 2001 John Wiley and Sons, Inc.

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Chapter 8

Preparation of Novel Delivery Agents for Delivery of Macromolecular Drugs Using Boric Acid Mediated Amidation of Carboxylic Acids and Amines

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Delivery agents comprising a carboxamide moiety are useful for the delivery of macromolecular drugs to the systemic circulation via oral administration. A practical synthetic pathway to these delivery agents has been developed by the direct amidation between a carboxylic acid and an amine using a catalytic amount of boric acid as a mediator. Boric acid is nontoxic, environmentally safe, and inexpensive. This method eliminates the preparation of acid chlorides, and is amenable for large-scale preparation providing excellent yields of the desired carboxamides. This boric acid mediated amidation between the carboxylic acids and amines with a variety of functional groups was achieved in a simple, single-step process using an equal molar mixture of acids and amines. This amidation works well with weakly nucleophilic amine, such as arylamines. The utility of the boric acid mediated amidation will be discussed.

Progress in the field of biotechnology has resulted in increasing number of novel macromolecular drugs with great clinical promise. Unfortunately, the delivery of these macromolecular drugs by routes other than parenteral is difficult. The delivery of such macromolecular drugs to the systemic circulation via oral administration has often been severely limited by biological, chemical and physical barriers, such as low pH in the stomach, powerful digestive enzymes, and impermeable gastrointestinal epithelium. Macromolecular drugs are easily rendered ineffective or destroyed in the stomach, by acid hydrolysis, and/or enzymatic degradation. Heparin, for example, by virtue of its anionic charge, high molecular weight, and hydrophilic nature, is not readily absorbed in the gastrointestinal tract (GI). To overcome the above-identified problems, specific compounds termed as delivery agents, or carriers, have been developed by Emisphere Technologies, Inc. These delivery agents are orally administered in combination with macromolecular drugs to facilitate the gastrointestinal absorption, and the subsequent delivery of the drugs.

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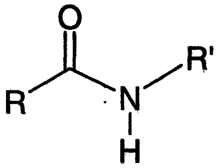
Carboxamides (formula 1) are known as a highly interesting class of compounds, and research directed to their formation is continuously attracting great interest in the synthetic organic chemistry community. Recently, the uses of carboxamides as delivery agents for the delivery of macromolecular drugs in a wide range of settings have gained great attention (*1,2,3*). One such example is the use of carboxamides for delivery of a protein drug, recombinant human growth hormone (rhGH) via oral administration (*3,4*). Since an increasing number of structurally diverse carboxamides are found to be useful for delivery of macromolecular drugs, our interest in this field is directed to a practical synthetic methodology of carboxamides to be used as delivery agents for oral delivery of macromolecular drugs.



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wherein R' is an aromatic group.

The chemistry of amide bond formation (amidation) is a vital chemical transformation in organic chemistry (*5*). Amide bonds are responsible for linking amino acids to form proteins. Despite many methods for the formation of carboxamides, new and effective methodologies are still needed. To develop a general program to prepare structurally diverse delivery agents of formula 1 for the delivery of macromolecular drugs via oral administration, we sought a facile method for the preparation of delivery agents comprising a carboxamide moiety.

The preparation of carboxamides **1** generally involves an amidation reaction between a carboxylic acid and an amine. Many different methods to accomplish this amidation are known. In most cases, a carboxylic acid is activated by conversion into a more electrophilic intermediate, like an acid chloride. The latter then reacts with an amine in the presence of an activating agent to form a carboxamide. While the method involving the acid chloride intermediate is commonly used for the preparation of carboxamides, it suffers severe difficulty and sometimes fails to work because many acid chlorides are unstable or not compatible with other functional groups present in the carboxylic acid and/or in the amine molecules. Therefore, before the conversion of a carboxylic acid into an acid chloride, the non-compatible functional groups present in the carboxylic acid molecule, such as hydroxy, thio, or amino groups must be protected. Once the protections have been accomplished, the acid chloride can be prepared and the subsequent coupling step can be carried out. The non-compatible functional groups must then be deprotected. Thus, the duration and the cost of the amidation increase because of the need of the protection / deprotection steps. Furthermore, the preparation of acid chlorides requires the use of hazardous materials such as thionyl chloride, oxalyl chloride, or phosgene. In addition, the volatile by-products generated from the reaction are highly corrosive. Coupling agents like DCC or EDC in the presence of 1-hydroxybenzotriazole, HOBt, HOAt (6) PS-HOBt, PS-HBTU, PS-TFP, HATU can also be employed in the amidation reaction of carboxylic acids. However, the by-products are not always easily removed, especially in large-scale preparations. There are limited reports in the literature about amidation using boron reagents such as boron trifluoride etherate (*7*), trimethylamine-borane (*8*), or tri-n-butylamine-borane (*9*), or other boron reagents (*10,11,12*) to provide carboxamides in moderate yields. However, the reported amidations need to employ at least one equivalent of boron reagents. Ishihara et al. disclosed direct amidations using boronic acid as a mediator, but only the boronic acids with strong electronic withdrawing substituents at the phenyl ring are efficient (*13*). In general, boronic acids are expensive, and the reported boronic acid (3,4,5-trifluorobenzeneboronic acid) is not commercially available at this time.

Recently, we have developed a practical and facile alternative synthetic pathway to effectively form an amide bond using boric acid as a catalyst. Direct amidation of carboxylic acids with amines using boric acid as a mediator has not been reported in the literature, and we wish to report it.

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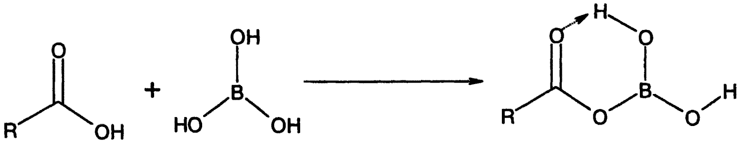
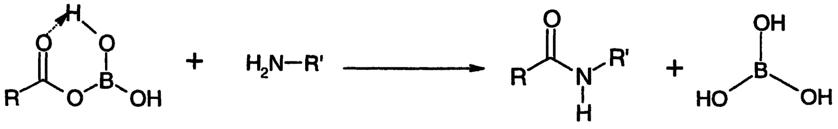
Reported herein is a new alternative synthetic method in which a carboxylic acid and an amine are coupled to form a carboxamide in the presence of a catalytic amount of boric acid. Mechanistically, we postulate that boric acid forms a reactive complex with the carboxylic acid: Acyloxyboron intermediate **2**.



2

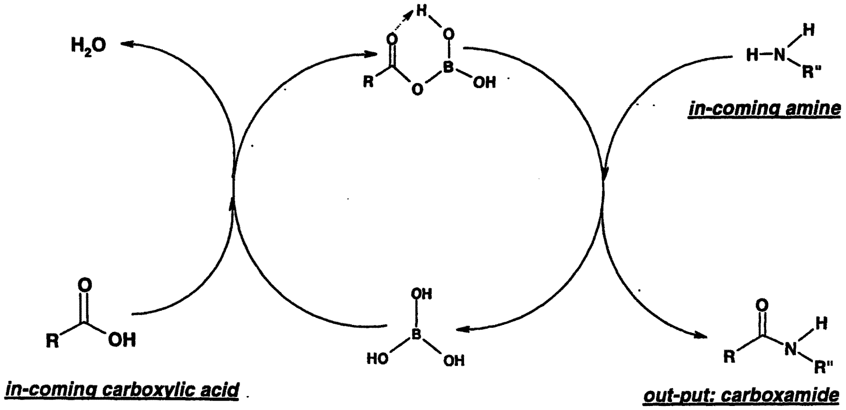
It is believed that a Lewis acid could enhance the rate of the formation of the **2** and the reactivity of the latter towards the amine in the reaction mixture. Upon reacting with an amine **3**, **2** yields the desired carboxamide **1** and regenerates boric acid, the amidation mediator (catalyst).



2 **3** **1**

The proposed mechanism for the boric acid-mediated amidation is summarized in the following catalytic cycle.

Catalytic Cycle



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To explore the utility and the scope of this boric acid mediated amidation, the catalytic reaction has been studied with structurally diverse carboxylic acids and phenylamines. The direct coupling reaction between carboxylic acids and phenylamines has conveniently been achieved in a single-step process using an equal molar mixture of acids and amines in refluxing toluene for 1 to 16 hours. Water is removed by means of a Dean-Stark separator. The versatility and the feasibility of this boric acid mediated amidation have been amply demonstrated by successful preparation of many carboxamides, useful for the delivery of macromolecular drugs. (see Table I).

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Results and Discussion

Several important generalizations emerge from the study of the boric acid mediated amidation of carboxylic acids and amines.

(1) In the absence of boric acid, no amidation is observed (Entry 13).

(2) In most cases, 5 mole % of the catalyst is sufficient to mediate the amidation.

(3) In contrast to several other methods for amidations employing coupling reagents including the use of boron reagents, transition metal catalysts (*14, 15, 16*), DCC, or EDC in the presence of 1-hydroxybenzotriazole (HOBt), HOAt (6) PS-HOBt, PS-HBTU, PS-TFP, HATU, the amidations require at least one equivalent of coupling reagent. In addition, those coupling reagents are only efficient with reactive amines. The present method is successfully applicable to aromatic amines, which are less nucleophilic and less reactive. This boric acid mediated amidation works very well with phenylamines, even bearing deactivating groups such as a carboxylic ester. We simply use 20 mole % of boric acid to drive the reaction to completion (17).

(4) In all cases, the reactions proceed cleanly in high yields to the expected carboxamides.

(5) No or little side reactions either with the unprotected hydroxy group present in the phenylamines(all the entries), or with the unprotected hydroxy group present in the carboxylic acids are observed (Entry 11). The catalytic amidations of unprotected α-hydroxycarboxylic acids also proceed well under similar conditions (18).

(6) The catalyst employed in the reactions, boric acid, is inexpensive and commercially available. The reaction is easy to conduct. Therefore, it is amenable for large-scale preparations.

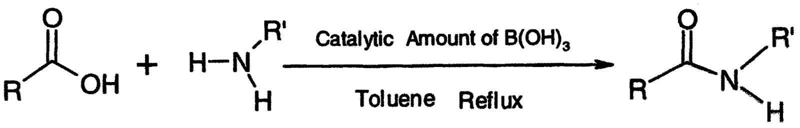


Table 1

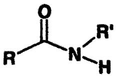
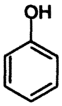
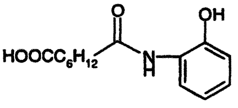
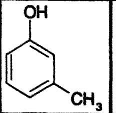
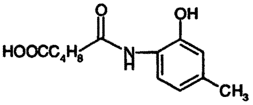
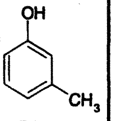
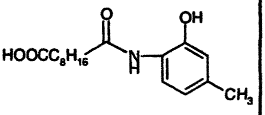
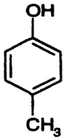
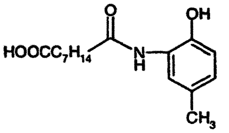
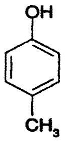
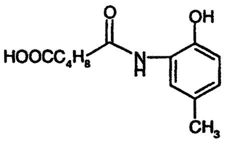
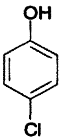
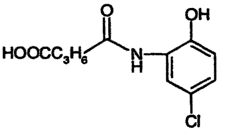
Entry	R (in acid)	R' (in amine)		Reaction Time (h)	Yield % After hydrolysis of the amidation product
1	$EtOOCC_6H_{12}$			16	75
2	$CH_3OOCC_4H_8$			4	77
3	$EtOOCC_8H_{16}$			4	78
4	$EtOOCC_7H_{14}$			4	77
5	$CH_3OOCC_4H_8$			4	78
6	$CH_3OOCC_3H_6$			16	72

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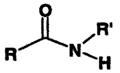
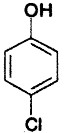
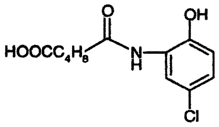
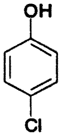
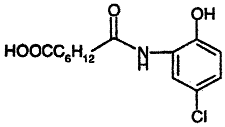
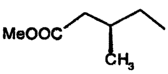
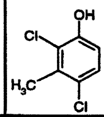
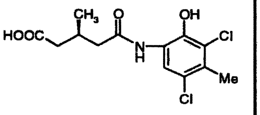
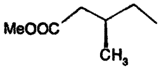
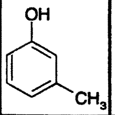
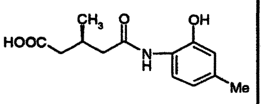
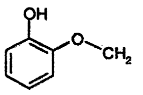
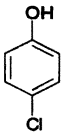
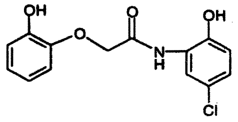
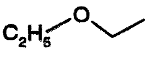
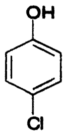
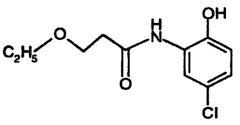
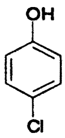
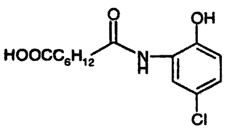
Table I (continued)

Entry	R (in acid)	R' (in amine)		Reaction Time (h)	Yield % After hydrolysis of the amidation product
7	$CH_3OOCC_4H_8$			16	70
8	$EtOOCC_6H_{12}$			16	57
9				16	68
10				16	70
11				4	92[a]
12				16	90[a]
13	$EtOOCC_6H_{12}$			16	0 [b]

(a) The yield of the direct amidation product.
(b) In the absence of boric acid.

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In conclusion, we have developed a highly efficient procedure to accomplish the direct amidation between carboxylic acids and amines using boric acid as a mediator. The novel methodology described here is complementary to existing methods of direct amidation known in the literature, and will find a variety of applications in synthetic organic chemistry and in medicinal chemistry. More importantly, this method is very practical in the field of delivery agents because so many carboxamides of de novo design, useful for the delivery of macromolecular drugs via oral administration, can be conveniently prepared using this facile single-step process.

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17. In a separate study with phenylamines bearing an ester group, the amidations went to completion when using 20 mole % of boric acid.
18. When an unprotected α-hydroxycarboxylic acid was employed, this boric acid mediated amidation proceeded well without significant ester formation.

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Chapter 9

Loosely Cross-Linked Silicone Elastomer Blends and Topical Delivery

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As a remarkable example of the emergence of polymers in the development of advanced pharmaceuticals, silicones have definitively proven their suitability for elaborating new therapeutic forms. Thanks to their biocompatibility and versatility, silicones are used in the preparation of various types of drug delivery systems, from simple creams to more complex transdermal patch or subcutaneous implanted devices. Topical applications would certainly benefit from the latest innovation in the silicone elastomer technology with *Loosely Cross-linked Silicone Elastomer Blends*. This chapter describes the application of loosely cross-linked silicone elastomer blends in topical drug delivery. Materials based on this technology were formed into elastomeric matrices and tested for estradiol release rates over 24 hours. These results were compared to other types of silicone materials tested in the same manner. The patches made from the loosely cross-linked silicone elastomer blends showed noteworthy 24-hour cumulative release rates of 45% to 58%. The other silicone materials tested showed 24-hour release rates that ranged from 1% to 12%.

Silicones and Drug Delivery Technology

Introduction

The fast emergence of macromolecular materials, derived from natural products or obtained from conventional chemical synthesis, has been accompanied by their wide introduction into all fields of medicine. The ability to control the molecular arrangements and the physico-chemical properties of polymers, the development of suitable processing techniques and equipments, and the possibility to prepare polymeric compositions with acceptable impurity profiles have allowed many polymers to be qualified as excipients in the commercialization of new pharmaceutical forms.

Indeed, since the 1960s, pharmaceuticals have come a long way from simple and fast-acting drug dosage forms to complex systems, allowing time and site control of active delivery. Better understanding of human body mechanisms, discoveries of new molecules, and innovative therapeutic treatments have certainly driven this advancement. But the increasing use of polymeric materials in the development and commercialization of new galenic forms has potentiated this progress, and made possible the construction of highly sophisticated devices and their application to new body areas (*1*).

On account of its biocompatibility, silicone is probably one of the most widely used polymers in medicine. The name silicone refers to compounds based on alkylsiloxanes, but the preferred structures for answering medical needs are the polydimethylsiloxane (PDMS) materials, which are currently used as antifoams in gastro-enterology or as excipients and process aids in pharmaceutical applications; some of them having now reached the status of pharmacopoeial compounds. Their use in drug delivery devices has been dramatically increased during the second half of the twentieth century, especially when the association of specific properties is critical to meet the requirements of the product design: biocompatibility, no sensitizing or irritating effect, suitable stability, permeability to moisture and gases, compatibility and permeability to selected molecules, processing flexibility, and ease of use (*2*).

New long-lasting drug delivery applications such as implant, insert, transdermal, or topical forms take advantage of the unique and intrinsic properties of silicones. Such controlled drug delivery devices allow controlled release of active molecules with biologically appropriate kinetics to the targeted area; they prevent the adverse effects - peak dosage, low compliance, or drug degradation - which are commonly observed with traditional oral and parenteral medication (*2*).

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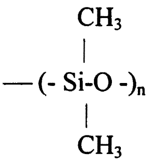
This chapter provides an overview of the polydimethylsiloxane materials used in pharmaceutical applications and explains how controlled drug delivery systems benefit from silicone technology. Estradiol is used as a drug example to illustrate these benefits, especially in topical formulations containing a new silicone elastomeric film former: the loosely cross-linked silicone elastomer blend technology.

Silicone Chemistry

Silicone is an umbrella term to designate materials based on organosiloxane chemistry, and more frequently it is an alternative name for polydimethylsiloxanes (Figure 1).



$$—(\text{-}\ \underset{\underset{CH_3}{|}}{\overset{\overset{CH_3}{|}}{Si}}\text{-}O\ \text{-})_n$$

Figure 1: Polydimethylsiloxane (PDMS)

Polydimethylsiloxanes have a semi-organic molecular structure made of a highly mobile siloxane backbone supporting a regular and non-polar arrangement of pendant methyl groups. They are synthesized by hydrolysis of methylchlorosilanes followed by condensation into cyclic or linear dimethylsiloxane oligomers, which are then polymerized into higher molecular weight molecules and also functionalized according to their intended uses as intermediates in the preparation of more complex formulations (*3*). The purity and reactivity of monomers (chlorosilanes and siloxane oligomers) and the use of catalysts that are easily eliminated allow maximizing the purity of polydimethylsiloxanes.

The mobility of the siloxane chain is truly unique, allowing an easy orientation of the polymer according to interactions with vicinal entities or surfaces. The methyl substitution is spread out at interfaces, forming a low surface energy "shield", which develops very low intermolecular interactions and unique surface characteristics. Thanks to the stability of Si-O-Si and Si-C bonds, silicones exhibit chemical inertness to many substances and are thermostable.

As a consequence of these molecular properties, silicones have unique behaviors. Because of low surface tension, they are able to wet virtually any

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surface and spread on a wide variety of substrates making them suitable for lubrication, coatings, and antifoam applications. They can be formulated to cover the broad range of rheological profiles from viscous fluid to visco-elastic thermoplastic (pressure sensitive adhesive) and rubber-like thermoset (elastomer). The low intermolecular interactions and the high intramolecular mobility provide outstanding anti-adherent property (useful to make release coating), further the permeability of silicones to various substances (including water vapor and drugs), and facilitate reactions between functional groups (substituted for methyls), allowing for fast ambient reactions (*4*).

Silicone in Pharmaceutical Applications

The pharmaceutical uses of polydimethylsiloxanes are widespread mainly due to the uniqueness and versatility of the dimethylsiloxane chemistry, which allows the preparation of various materials offering a wide range of physical properties: fluids from volatile to very high molecular weight gum, surfactants, functionalized resins, compounds and pressure sensitive adhesives, and fillerless to highly reinforced elastomers.

The polydimethylsiloxanes, including fluids, compounds, and emulsions, specified in the pharmacopoeia monographs "Dimethicone", "Silicone Oil," and "Simethicone" are mainly used as siliconizing agents, lubricants, antiflatulents in gastro-enterology, defoaming agents of biological fluids, as topical excipients to enhance substantivity and bio-disponibility, or process aids in pharmaceutical manufacturing operations (*2*).

Loaded with silica and/or cross-linked, the polydimethylsiloxanes are turned into fillerless elastomers or higher durometer rubbers. The hydrosilylation addition is the preferred cure system for pharmaceutical applications (Figure 2). This reaction requires a very low level of platinum catalyst and does not generate any by-products. The cross-linking reaction can either be completed at ambient or accelerated at higher temperatures; they are typically complete within a few minutes even at room temperature, and take place *in situ* in a body cavity such as the buccal sphere or an open wound.

$$\equiv Si\text{--}CH=CH_2, + H\text{--}Si\equiv \xrightarrow{\text{Platinum complex}} \equiv Si\text{--}CH_2\text{-}CH_2\text{--}Si\equiv$$

Figure 2: hydrosilylation reaction

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A fillerless elastomeric formulation based on hydrosilylation cure chemistry, as described above, can be prepared and cross-linked in low viscosity polydimethylsiloxane to produce *Loosely Cross-linked Silicone Elastomer Blends* or *LCSEBs*. The three-dimensional network is formed by the cross-linking reaction occurring in a diluted state. This material can be further sheared and dispersed into a silicone fluid, which swells the elastomeric particles to form a homogeneous gel. According to the elastomer formulation and the dispersing fluid, various elastomer gels can be prepared to deliver specific properties. They are currently used in personal care applications to provide or improve spreadability, substantivity, silky feeling, thickening effect, or active delivery (*5*). These materials are further described later in the chapter.

Reinforced and tackified with siloxane resin, the polydimethylsiloxanes gain a thermoplastic 3D structure while remaining soluble in non-polar solvents, and a visco-elastic rheological profile making them suitable *pressure sensitive adhesives* (*PSAs*) for skin applications. To be used in healthcare applications, PSAs have to comply with some specific requirements, the most critical being biocompatibility, skin compliance when adhering or at removal, stability, permeability to drugs and moisture, and the ability to be co-formulated or easily processed. Silicone PSAs, thanks to their intrinsic properties, have been recognized for 30 years as suitable for prolonged skin contact applications and have been used in various transdermal drug delivery systems (*6*).

Controlled Drug Release Systems

Drug therapy is commonly influenced by the timing of the administration, the dosage, and the route of dosing. Classical pharmaceuticals (tablet, syrup and injection) require recurring high dosage to maintain an effective level of active over a suitable period of time. Controlled drug delivery to the blood stream via permeation through body tissues allows avoiding sharp sinusoidal treatment by maintaining a constant, therapeutically effective and prolonged flow of drug to the systemic circulation or directly to targeted treated tissue, while by-passing GI tract degradation or hepatic first-pass metabolism. Such controlled drug delivery systems are implant, insert, mucoadhesive, transdermal or topical therapeutic forms.

Transdermal drug delivery systems consist of drug containing adhesive patches, which adhere to intact skin up to 7 days. The patch design controls the release of active, which is then carried through the organism by the blood circulation for a systemic activity. Using also the skin as entry point, the topical forms are used for local treatment (muscle or skin diseases), within a shorter period of time; they consist of an adhesive plaster or in a film-forming and substantive material (e.g. cream or gel).

There are two main types of diffusion-controlled release devices: the "matrix type device", where the active is dispersed in a polymer matrix, and the "reservoir-membrane device", where the active is encapsulated behind a rate-controlling polymeric membrane. These designs might have a relatively high level of complexity according to the dosage form and delivery location; they must contain an excess of drug (i.e. over the saturation level) to ensure a stable release over a long period of time. They differ by their release and kinetics profiles (*7, 8*). The matrix approach is favored when a short and quick delivery is required, for instance in acute treatments. The matrix material is formed in a solid drug release device or formulated to be an in situ film-forming product. In the reservoir type device, the active is separated from the biological tissue by a rate-controlling membrane; the release rate is constant versus time unlike in the matrix in which the release slowly decreases with time.

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Silicones to Control the Release of Estradiol

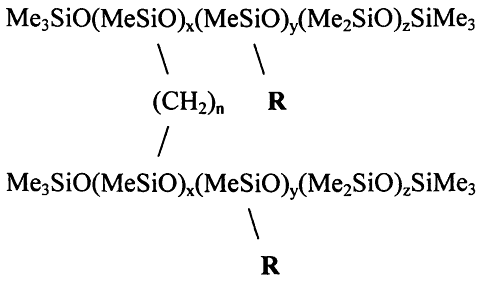
The effectiveness of silicone rubber, as capsule or matrix, to control the release of actives was described in the early 1960's. These early efforts highlighted the ability of these systems to deliver hormones, demonstrating a better monitoring of the dosage and an improved safety over other forms (*9, 10, 11*). Ever since, the concept of using silicone elastomers as rate-controlling membranes or matrices has been studied in detail to determine the factors that significantly impact the permeation of estradiol, for example, through a silicone-based network (e.g., lattice density and reinforcing filler nature, permeation enhancer and co-solvent, hydrophilicity-lipophilicity balance of estradiol derivatives) (*12, 13*). The use of silicone rubber in the construction of estradiol delivery devices for fertility control and hormone therapy in humans is now well documented and widely used by the pharmaceutical industry as long-term implants, inserts, or transdermal patches (*14 - 25*).

Silicone PSAs are efficient adhesives to maintain in place the therapeutic patch while allowing for the estradiol to permeate and be released at the appropriate rate (*26, 27)*. Silicone materials, rubber, or PSAs, can be co-formulated with pharmaceutical excipients to adjust the release profile of the drug to the intended application, or improve the permeability of the active molecule through the tissues to the targeted site (*28 - 30)*. Using the drug estradiol, several types of silicone materials, including the loosely cross-linked silicone elastomer blends, were recently tested for cumulative drug release and for performance as drug delivery vehicles.

Loosely Cross-linked Silicone Elastomer Blend Technology

Chemistry

Until the early 1990's, essentially all of the silicones used in cream and lotion formulations for topical application were liquids such as dimethicone and cyclomethicone, or silicone resins, which in pure form are brittle solids. This changed with the introduction of loosely cross-linked silicone elastomer blends (*LCSEB*s), a new class of silicone ingredients that are based on cross-linked silicone polymers (5). These silicone elastomers are soft solids with aesthetic properties unlike any other class of silicones.



$$\begin{array}{l} Me_3SiO(MeSiO)_x(MeSiO)_y(Me_2SiO)_zSiMe_3 \\ \qquad\qquad\quad \backslash \qquad\quad \backslash \\ \qquad\qquad\quad (CH_2)_n \quad \mathbf{R} \\ \qquad\qquad\quad / \\ Me_3SiO(MeSiO)_x(MeSiO)_y(Me_2SiO)_zSiMe_3 \\ \qquad\qquad\qquad\qquad\quad \backslash \\ \qquad\qquad\qquad\qquad\quad \mathbf{R} \end{array}$$

Figure 3. Basic chemical structure of loosely cross-linked silicone elastomer blends. These materials are swollen in cyclomethicone or any other suitable liquid.

LCSEBs are comprised of lightly cross-linked siloxane chains swelled in diluents such as cyclomethicone. Prior to cross-linking via hydrosilylation, the siloxane chains can be varied by length and by the amount of reactive (SiH) sites versus non-reactive sites (Si-methyl). The siloxane chains can also be functionalized with various groups, such as polyether or alkyl. The remaining reactive sites are then connected by cross-linking with α,ω-dienes. By altering the ratios of the components (impacting the degree of cross-linking, amount of functionality, degree of dilution, etc.), the material properties can be greatly impacted. What is unique about the manufacture of these materials is that they are cross-linked in a diluted state, thereby producing a very loose cross-linked network. After cross-linking, the materials can be further diluted with cyclomethicone, or other suitable solvents, to the desired viscosity and texture. Unlike conventional siloxane polymers, they are not soluble in any solvent, but swell exceedingly in the presence of a suitable solvent. Materials formed can

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range from pourable liquids to very stiff, crumbly gels to creamy pastes. This versatility enables targeting specified properties and designing the silicone elastomer to meet specific needs. Another advantage of the loosely cross-linked silicone elastomer blends is that they can be dried down into elastomeric films if the diluent is volatile. The general structure is illustrated in Figure 3, with *Me* designating methyl groups, *R* designating functional groups such as polyether or alkyl, and $X \geq 2$.

Compatibilities

Formulation compatibility has been demonstrated with both polar and non-polar organic oils, waxes, polar solvents, etc. Because functional groups can be added to the siloxane chains prior to cross-linking, compatibilities with diverse materials can be targeted. This enables broad formulation parameters and permits a large variety of delivery matrices (*31*).

Without using additional surfactants, simple water-in-oil emulsions have been prepared from polyether modified silicone elastomer blends. Multiple emulsions are also possible (oil-in-water-in-oil as well as water-in-oil-in-water), enabling the delivery of either hydrophobic or hydrophilic actives from the resulting matrices (*32*).

Actives that have been formulated into emulsion delivery systems include oil soluble vitamins, such as vitamin A acetate, vitamin A palmitate, and vitamin E, water-soluble vitamins (C), sunscreens (octylmethoxycinnamate and octyl salicylate), antiperspirant salts (*33*), and estradiol, the drug chosen for testing.

The Testing of other Silicone Materials

Initially, experimentation was conducted to develop an understanding between polymer structure/composition and the effects of additives on the physical characteristics of silicone materials. The objective was to characterize various silicone materials for the controlled release of estradiol. Results of estradiol release from the following Dow Corning silicone materials are provided for comparison to the loosely cross-linked silicone elastomer blends.

- In situ curable silicone elastomer dispersed in a volatile silicone. It contains 17 wt% silica dispersed in a network made of 60,000 mPa.s viscosity PDMS. Based on a condensation cure, it yields a 45 Shore A durometer elastomer.

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- Low consistency flowable silicone elastomer. It contains 24 wt% silica dispersed in a network made of 2,100 mPa.s viscosity PDMS. It is based on a hydrosilylation cure, which yields a 25 Shore A durometer elastomer.
- Liquid Silicone Rubber (LSR) – It contains 28 wt% silica dispersed in a network made of 55,000 mPa.s viscosity PDMS. It is based on a hydrosilylation cure, which yields a 30 Shore A durometer elastomer.
- Silicone Latex – Water dispersion of silicone elastomer based on a hydrosilylation cure. The silicone elastomer is made of 10 wt% silica dispersed into a network of 55,000 mPa.s viscosity PDMS.
- Soft Skin Adhesive – Fillerless silicone elastomer (or gel) made via hydrosilylation. It is based on a chain extended silicone network of 450 mPa.s viscosity PDMS.
- Silicone Pressure Sensitive Adhesive (PSA) – High tack amine compatible silicone PSA, made of 55 wt% silicate resin, condensed with 13,500 mPa.s viscosity PDMS fluid.

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Test Methodology

Membrane/Patches

All formulations were fabricated into matrix patches. The loosely cross-linked silicone elastomer blends were formed into patches by drawing out a drug-loaded diluent-swollen elastomeric silicone material onto a Scotchpak® release liner using 30 mil (0.75 μm) shims, and drying for 1 hour in a 65°C oven. The film was covered with another sheet of Scotchpak® release liner and punched into 4 cm diameter patches using a Carver press. Silicone latex, Soft Skin Adhesive, silicone elastomers, and PSA formulations were cast on polyester release liner, allowed to devolitalize or cure, laminated with a polyester backing and cut into 4 cm diameter patches for testing. Dow Corning® LSR formulations were pressed cured between sheets of Teflon® liner.

Drug Permeation

Characterization of drug release from the matrix patches was performed on an eight-cell Franz Diffusion System without the use of a controlling membrane. Estradiol analysis was performed using High Pressure Liquid Chromatography (HPLC). A solution of Polyethylene Glycol 400 and deionized water 40:60 (v/v) was selected as the receptor media to obtain suitable estradiol solubility; estradiol solubility in water or saline alone was found to be less than 10 μg/ml. Sampling times were established at one, two, four, six, eight, and twenty-four hours. The receptor fluid was completely replaced at each point of sampling. HPLC analysis for estradiol was performed immediately after the completion of the diffusion experiment. Estradiol standards of known concentration were run before and after each set of samples to calibrate the instrument, quantitate the samples, and verify the instrument's condition.

Test Results

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It was theorized that the loosely cross-linked matrix would make these gels ideal carriers for actives/drugs. Four variations of *LCSEBs* were prepared in order to test the effects of variations on estradiol release rates. Variations targeted for testing included polyether functionality attached to the siloxane molecules, and estradiol point of addition into the silicone network.

Polyether functionality was chosen primarily to verify how a higher hydrophilicity and an increased polarity of the silicone network would impact the flow of estradiol out from the matrix. According to the physical and processing characteristics of materials, the active could be added prior to cross-linking or post-added. With most of the elastomeric silicones (samples E, F, G, and I in Table II), the drug loading was done prior to the cross-linking because the active could not be easily and thoroughly dispersed into the thermoset network even by swelling it with a solvent. In the case of materials dispersed in water or solvent, such as *LCSEBs* (in cyclomethicone), silicone latex (in water, sample H) and silicone PSA (in heptane, sample J), the drug was loaded into the final materials, which were then coated and dried into a thin film. *LCSEBs*, due to the cure nature of the material, allowed for both drug-loading processes. Thus, the option was available to add the estradiol to the final materials, as they had the consistency of smooth pastes, and the drug could easily be dispersed uniformly into the swollen elastomer.

The tested materials included four variations of *LCSEBs*, which varied the type of polyether, and the point of addition of the estradiol (Table I). Except for these intended variations, the samples were made under the same conditions, using the same raw materials. The estradiol loading is based on the elastomer, or non-volatile content of the blends.

Figure 4 illustrates the estradiol release rates versus time for the *LCSEBs*, converted to percentage of total drug loaded into each sample. In the case of all

Table I. Test Samples: different variations of loosely cross-linked silicone elastomer blends, and level of estradiol loading

Sample	*Material Tested*	*Estradiol*
A	Estradiol post-added to silicone elastomer with no substitution	5%
B	Estradiol pre-added: silicone elastomer with 2.7% EO_7 polyether	5%
C	Estradiol post-added: silicone elastomer with 2.7% EO_7 polyether	5%
D	Estradiol post-added: silicone elastomer with 5.4% EO_{12} polyether	5%

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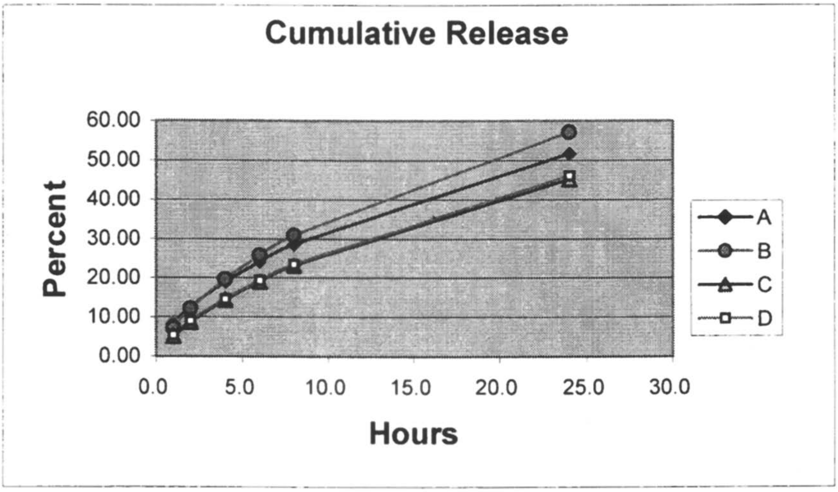

Figure 4. 24 hour cumulative release (CR) of estradiol from loosely cross-linked silicone elastomer blend samples A, B, C, & D.

four samples, the estradiol release rates for 24 hours were much higher than other silicone materials. Figure 5 illustrates the typical release rates for the other categories of silicone materials that were tested. The 24-hour cumulative release (CR) for these materials ranged from 1.0% up to 11.7%, compared to the four loosely cross-linked silicone elastomer materials (Figure 4), where the release rates range from 45% to 58%. This release was achieved without the use of additional drug release excipients or enhancers. The data for these graphs are listed in Table III.

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Table II. Test Samples: different variations of silicone materials.

Sample	*Material Tested*	*Estradiol*
E	In situ curable silicone elastomer dispersion	5%
F	Low consistency silicone elastomer	5%
G	Liquid silicone rubber 30 Shore A	5%
H	Silicone latex	5%
I	Soft skin adhesive	5%
J	Silicone PSA (high tack, amine compatible)	5%

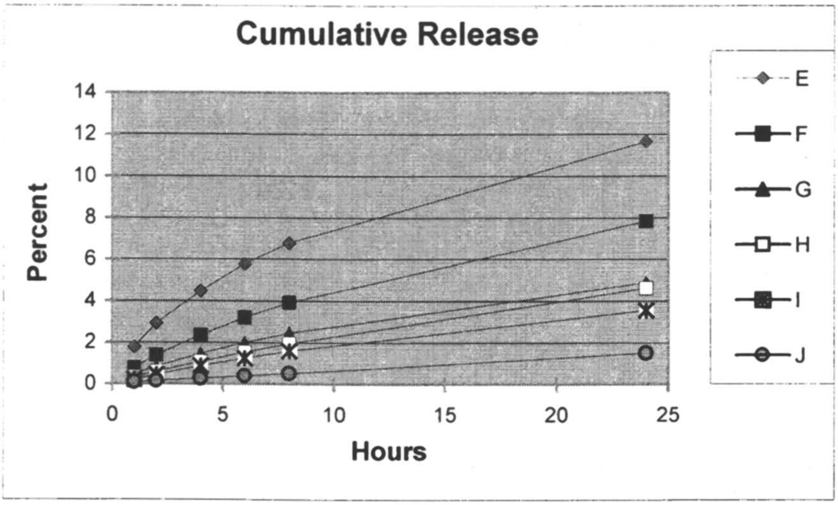

Figure 5. 24 hour CR of estradiol from various silicone materials.

Table III. Cumulative release of estradiol for test samples.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*
Time	*Cumulative Release Converted to %*									
(hr)	%	%	%	%	%	%	%	%	%	%
1	8.56	7.31	5.06	5.43	1.78	0.77	0.46	0.32	0.22	0.09
2	12.63	12.34	8.68	9.00	2.93	1.38	0.84	0.59	0.45	0.15
4	18.95	19.92	14.33	14.62	4.47	2.36	1.46	1.07	0.86	0.27
6	24.04	26.16	19.05	19.37	5.78	3.20	1.99	1.53	1.24	0.39
8	28.60	31.40	23.08	23.46	6.81	3.93	2.45	1.94	1.59	0.51
24	51.72	58.07	45.20	46.05	11.67	7.84	4.88	4.62	3.57	1.50

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Results and Discussion

It can be concluded from this study that polyether substitution on the loosely cross-linked silicone elastomer blend materials does not greatly impact the 24 hour CR, nor does increasing the amount or chain length of the polyether. In fact, according to the data, the polyether might actually slow the rate of release (Figure 4, sample A versus samples C & D).

The results also indicate that the point of estradiol loading may influence 24-hour cumulative release; the sample with the highest CR is the sample where the estradiol was added prior to cross-linking (Figure 4, sample B). NMR data indicate that a portion of the estradiol actually chemically bonds to the elastomer moiety when added at this step in the process. Yet, when tested, this sample had a 24 hour CR of 58% versus 45% for a similar sample where the estradiol was post-added (see Table III, sample B versus sample C). Unlike the polyether groups grafted to the silicone network, grafted estradiol might actually assist in the release of free estradiol from the matrix. It should be noted at this point that the estradiol/silicone elastomer blend material (sample B) has not been tested or approved for human testing, and that it was investigated as a research only sample under in vitro testing conditions.

The ability to post-add estradiol to the *LCSEBs* does have an advantage over the thermoset silicone materials tested, where adding the drug prior to cross-linking was required.

The most significant point of this study is that *LCSEBs* demonstrate significantly higher 24 hour CR over the other silicone materials tested. These results support the hypothesis that the degree of tightness of the cross-linking of a silicone matrix directly affects the drug release rate (*34*). Materials with higher lattice tightness due to filler reinforcement or cross-link density likely bind up the drug into the matrix, and hinder its release.

The high level of release rate distinguishes the loosely cross-linked silicone elastomer blends from the other silicone materials. This characteristic could be beneficial for applications where a high level and steady release rate is desired within a short period, such as a few days. The loosely cross-linked silicone matrix acts as a sustained drug released reservoir, which should allow for a better bioavailability of the estradiol, likely due to a higher diffusion rate and a more intimate interface between the topical matrix and the stratum corneum. This could also allow for minimizing the amount of drug needed in the formulation, and provide solutions for cost-effective formulation in terms of manufacturing process and dosage efficiency.

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Chapter 10

Hydrogels by Stereocomplex Formation and Their Use as Drug Delivery Matrices

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Block and graft copolymer hydrogels, physically crosslinked through stereocomplex formation of poly- or oligo(lactic acid) chains of opposite chirality, are described. Protein-loaded hydrogels of dextran-*g*-oligo(L/D-lactic acid) are obtained from aqueous solutions of the two polymers (containing L- and D-grafts, respectively). These hydrogels degraded in a period of 1-7 days, depending on polymer composition. Lysozyme was quantitatively released with full preservation of its activity.

In Advances in Controlled Drug Delivery; Dinh, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Hydrogels are polymeric networks, which absorb and retain large amounts of water. In general, hydrogels possess a good biocompatibility. Their hydrophilic surface has a low interfacial free energy in contact with body fluids, which results in a low tendency for proteins and cells to adhere to these surfaces. Moreover, the soft and rubbery nature of hydrogels minimizes irritation to surrounding tissue. Therefore, hydrogels have found widespread application in different technological areas, e.g. as materials for contact lenses and protein separation, matrices for cell-encapsulation and devices for the controlled release of drugs and proteins.(*1–5*)

For many applications, such as drug delivery, it is advantageous that the hydrogels are biodegradable. Labile bonds can be present either in the polymer used to prepare the gel or in the crosslinks. These bonds can be broken under physiological conditions, in most of the cases by hydrolysis, either enzymatically or chemically.(*5*) It is of great interest to have control over the degradation kinetics; in other words, to have control over the parameters by which the degradation characteristics can be tailored. Moreover, once the hydrogels are implanted it is important that the formed degradation products have a low toxicity meaning that the formed compounds can either be metabolized into harmless products or can be excreted by the renal filtration process. The nature of the formed degradation products can be tailored by a rational and proper selection of the hydrogel building blocks.

Both chemical and physical methods have been used to create hydrogels.(6) In chemically crosslinked gels, covalent bonds are present between different polymer chains. In physically crosslinked gels, dissolution is prevented by physical interactions, which exist between different polymer chains. In recent years, there is an increasing interest in physically crosslinked gels, especially in which the gel formation occurs under mild conditions in the absence of organic solvents. The main reason is that the use of crosslinking agents and organic solvents to prepare such hydrogels is avoided. These agents and solvents can not only affect the integrity of the substances to be entrapped (e.g. proteins, cells), but they are often toxic compounds which have to be removed/extracted from the gels before they can be applied. To create physically crosslinked gels a great variety of methods have been applied, including ionic, hydrophobic and hydrogen bond interactions.(6) Also the formation of crystalline domains is a tool to create physical crosslinks. The latter includes the formation degradable stereocomplexes, which is the subject of this contribution. In this context, we will describe our newly developed biodegradable hydrogel system based on biocompatible substances, *i.e.* dextran and lactic acid oligomers. These hydrogels can be prepared from pure aqueous solutions and can entrap and release proteins and enzymes without affecting their integrity.

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Macromolecular Stereocomplexes

In low molecular weight compounds possessing a chiral center, the formation of racemic crystals upon mixing the two enantiomers is a well-known phenomenon. A higher melting temperature (T_m) is frequently observed for the racemic crystallites than for each of the enantiomers. For example, D- and L-tartaric acid have a T_m of 173 °C, whereas for its racemic mixture a T_m of 206 °C is detected.(*7*)

In polymers of opposite chirality the formation of racemic crystallites has also been observed. In the literature, the formation of such racemic crystallites has been referred to as stereocomplexes and was first described by Dumas *et al.* in 1972.(*8*) They reported a melting point of 165 °C for an optically active poly(*t*-butyl-thiirane) as compared to 205 °C for the corresponding blend of poly(*R*-*t*-butyl-thiirane) and poly(*S*-*t*-butyl-thiirane). The difference in melting temperature was due to different crystal structures, as reported by Matsubayashi *et al.*.(*9*) Stereocomplexes were also observed for mixtures of the R- and S-forms of poly(α-methylbenzyl methacrylate),(*10*) poly(α-methyl-α-ethyl-β-propiolactone) (PMEPL),(*11*) poly(γ-benzyl glutamate),(12) poly(β-benzyl aspartate),(*13*) and α-olefin-carbon monoxide poly(1,4-ketone)s.(*14*)

It should be noted that the term stereocomplex is not exclusively used for racemic crystallites formed by chemically identical polymers of opposite chirality. Earlier, this term was used to describe the interaction between syndiotactic and isotactic polymers.(*15, 16*) Since these polymers do not have the same chemical structure, the term stereo-selective complexes, as suggested by Lohmeyer *et al.* is to be preferred in these cases rather than the term stereocomplexes.(*17*)

PLA Stereocomplex

Poly(lactic acid) (PLA) is a polyester, which consists of repeating units of lactic acid. Lactic acid, 2-hydroxypropionic acid, contains a chiral center and can therefore be in the L- or D-configuration. PLA is usually obtained by ring-opening polymerization of lactide, the cyclic dimer of lactic acid. Bulk polymerization of lactide with retention of stereochemistry can be carried out in the melt at 130 °C in the presence of the catalyst tin octoate.(*18, 19*) PLLA and PDLA, the homopolymers of L-lactic acid and D-lactic acid respectively, are semicrystalline materials. High molecular weight PLA, of either stereoisomer, has a melting temperature (T_m) of 170 °C, a melting enthalpy (ΔH_m) of 70 J/g, and a glass transition temperature (T_g) of 60 °C.(*20*) In blends of high molecular weight PDLA and PLLA, a phase with a higher T_m (230 °C) is observed. This phase is ascribed to stereocomplex formation. Racemic crystals were also

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observed for the monomer lactide: the melting point (T_m) of D,D-lactide or L,L-lactide is 95 °C, whereas the 1:1 racemate of D,D-lactide and L,L-lactide has a higher melting point of 126 °C.(*21*) The ability of PLA to form stereocomplexes was first described in a patent publication by Murdoch and Loomis,(*22*) and the first paper on these complexes was published by Ikada *et al.*(*23*)

By X-ray structure analysis, the unit cell of the crystals in the homopolymers of lactic acid was found to contain two 10_3 helices; poly(L-lactide) consists of left-handed helical chains and poly(D-lactide) of right-handed helical chains.(*24*) In the stereocomplex crystal, a poly(L-lactide) segment and a poly(D-lactide) segment are packed side by side in a 1:1 ratio of L and D monomer units and packed laterally in parallel fashion.(*24*) The unit cell of the complex contains three L- and three D-monomer units of the PLLA and PDLA helices, which can be packed more densely than left-handed or right-handed helices alone. Each PLLA and PDLA forms a more compact 3_1 helix in the complex crystal.(*24–26*) The following mechanism of growth for the triangular lamellar stereocomplex crystal was suggested by Brizzolara *et al.*:(*27*) As crystallization starts, for example, one PDLA helix will be surrounded by three PLLA helices. Because of the triangular shape of the 3_1 helix, a triangular nucleus is thus formed whose respective sides are built up exclusively by PLLA. In the next step a PDLA layer grows on the crystal structure and then again a PLLA layer grows onto the PDLA layer and so on. Van der Waals forces between the helices cause a specific energetic interaction-driven packing of the helices. These interactions cause the higher stability and consequently the higher melting point of the stereocomplex.(*27*)

PLA stereocomplexes were studied extensively as a new class of biodegradable materials with higher mechanical strength, improved thermal stability, and less sensitivity to hydrolysis than synthetic polyesters such as poly(glycolic acid) and PLA.(*28*) Stereocomplex PLA fibers were prepared by spinning from a mixed solution of PDLA and PLLA, and by dry spinning from a melt of PDLA and PLLA to obtain reinforced materials, which are stronger than PLLA.(*29*) However, PLA stereocomplexes have some drawbacks. They are highly resistant to degradation and thereby adversely affect the biocompatibility.(*30–33*) The poor biodegradation and biocompatibility of the PLA stereocomplexes resulted in a decreased interest in these systems. Nevertheless, at present stereocomplexes have regained interest for application in drug delivery systems.

Hydrogels Based on Stereocomplex Formation

Stereocomplex formation between PLLA and PDLA, as described above, has been applied by several groups for the preparation of biodegradable

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hydrogels. The general feature of these hydrogels is that polymers or oligomers of either L-lactic acid or D-lactic acid are attached to a water-soluble polymer in the form of block or graft copolymers. Association takes place in crystalline domains (stereocomplexes) upon mixing the two polymers (one containing L-lactic acid, the other containing D-lactic acid), providing the physical crosslinks. We would like to classify PLA stereocomplex hydrogels as follows:

1. Hydrogels containing high molecular weight (HMW) PLA chains. The individual enantiomeric polymers (PLLA and PDLA) are already crystalline and mostly insoluble in water. Mixing should therefore take place from organic solutions or in the melt, and the resulting blend can subsequently be swollen in contact with water. The difference of these stereocomplex hydrogels with respect to hydrogels containing PLLA or PDLA alone is that the crystalline domains are more stable and resistant to hydrolytic degradation.
2. Hydrogels containing oligomeric lactic acid (OLA) chains, whose individual enantiomeric polymers (OLLA and ODLA) can be soluble in water when the lactic acid content is sufficiently low. This provides the unique opportunity to form hydrogels by stereocomplex crystallization from aqueous solutions.

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Stereocomplex Hydrogels Containing HMW PLA.

Stereocomplex formation between triblock copolymers of PLLA-PEG-PLLA and PDLA-PEG-PDLA (PEG = poly(ethylene glycol)) was studied with the aim to prepare hydrogels.(*34*, *35*) The release of bovine serum albumin (BSA) from microspheres based on these triblock copolymers, has been studied by Lim *et al.* and compared with the release of BSA from microspheres prepared with one enantiomeric form of the triblock copolymer and with PLA microspheres.(*34*) The protein-loaded microspheres were prepared by a double-emulsion solvent evaporation method. The stereocomplex and single enantiomeric triblock copolymer microspheres showed a slightly larger burst release than PLA microspheres, which is likely caused by the higher water-uptake capacity of the PEG-containing microspheres. Although the morphology of the stereocomplex microspheres was clearly deviating, the release of BSA was similar to the single enantiomeric triblock copolymer microspheres.

Stereocomplex Hydrogels Containing OLA.

The triblock copolymers mentioned in the previous section are water-soluble when the hydrophobic PLA blocks are sufficiently short. The maximum length of the lactic acid blocks for rendering water-solubility decreases with decreasing length of the PEG blocks used. For example, PEG-block with a molecular weight of 13.000 g mol^{-1} substituted with on average 26 lactic acid repeating units on each side of the PEG block are water-soluble and can form stereocomplexes upon mixing of aqueous solutions of each enantiomer.(*35*)

Recently, another system has been prepared by Lim *et al.*, based on stereocomplex formation by enantiomeric oligo(lactic acid) (OLA) side chains grafted onto poly(2-hydroxyethyl methacrylate) (PHEMA) (poly(HEMA-*g*-oligolactate)s).(*36*) The system was prepared by casting a film from poly(HEMA-*g*-oligo(L)lactate) and poly(HEMA-*g*-oligo(D)lactate), both dissolved in chloroform. Among other characteristics, the degradation of the obtained film was compared with the degradation of a film cast from a solution of a single enantiomer of the graft copolymer. Slower degradation was observed for the 1:1 blend of the L- and D-forms than for the single enantiomer. Stereocomplex formation from water was not investigated and is most likely not possible due to the high grafting density.

We realized the importance of avoiding organic solvents for the dissolution of the individual enantiomeric polymers to be used for the formation of stereocomplex hydrogels when aiming at biomedical applications such as the delivery of pharmaceutically active proteins. Therefore, we prepared biodegradable and biocompatible hydrogels based on dextran (a natural occurring polysaccharide) grafted with OLA and investigated the minimum and maximum length of the grafts required to form stereocomplexes after mixing and retaining water-solubility before mixing, respectively. The results and the application as a protein delivery device will be summarized in the next section.

Dextran-g-OLA Stereocomplex Hydrogels

In our Department we designed a hydrogel system based on dextran in which crosslinking is established by stereocomplex formation between lactic acid oligomers of opposite chirality. First, we investigated whether an 'operation window' of lactic acid chain lengths is present, in which stereocomplex crystallization would occur whithout homocrystallization of the individual enantiomers. Therefore, we isolated monodisperse lactic acid oligomers by preparative HPLC, from a polydisperse mixture obtained by conventional ring opening polymerization of L- or D-lactide. It was shown that crystallinity was present in individual D- or L-oligomers with a degree of polymerization (DP, *i.e.* the number of lactic acid repeating units) ≥ 11. On the other hand, in blends of D- and L-oligomers of lactic acid crystallinity (stereocomplexation) was already observed at a DP ≥ 7 (see Figure 1).(*37*)

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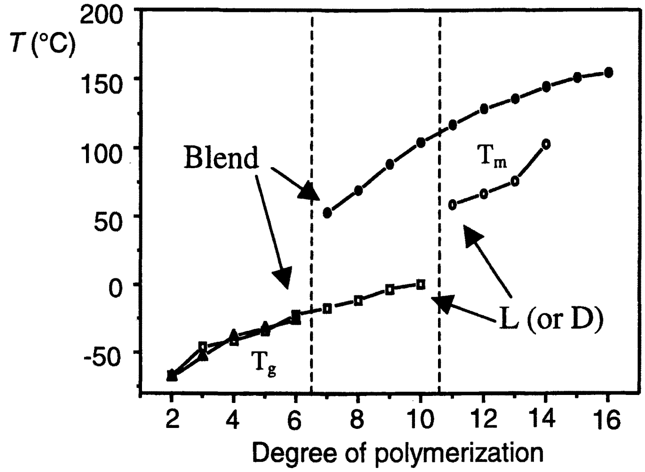

Figure 1. Glass transition (T_g) and melting temperatures (T_m) of lactic acid oligomers from differential scanning calorimetry measurements: OLLA (or ODLA) (open symbols) and 1:1 mixture of OLLA and ODLA (filled symbols). (Adapted with permission from reference 37. Copyright 1998 American Chemical Society.)

In the next step, polydisperse or monodisperse L- and D-lactic acid oligomers were coupled via their terminal hydroxyl group to dextran, yielding dextran-*g*-OLLA and dextran-*g*-ODLA, respectively, with variation in DP of the oligolactic acid and degree of substitution (DS, percentage of substituted dextran repeating units) (Figure 2). Interestingly, each product was soluble in water separately and upon mixing solutions containing OLLA- and ODLA-grafted dextran, hydrogels are formed at room temperature as demonstrated by rheological measurements.(*38*) As can be seen in Figure 3, the storage modulus of the obtained hydrogel strongly decreased upon heating to 80 °C, while it was restored upon cooling to 20 °C demonstrating the thermo-reversibility and the physical nature of the crosslinks. The storage modulus of the gels depends on the degree of polymerization of the lactate acid grafts and their degree of substitution on dextran. Mixtures of dextran-*g*-OLLA and dextran-*g*-ODLA containing monodisperse grafts with a DP lower than 11 did not result in a hydrogel. This is in contrast to the observation that stereocomplexation already can occur for non-grafted OLA chains with a DP $\geq$ 7. This difference can be explained by hampered stereocomplex formation once the oligomers are both coupled via their hydroxyl group to dextran (Figure 4A). Interestingly, gel formation was favored when one lactic acid oligomer was coupled via its

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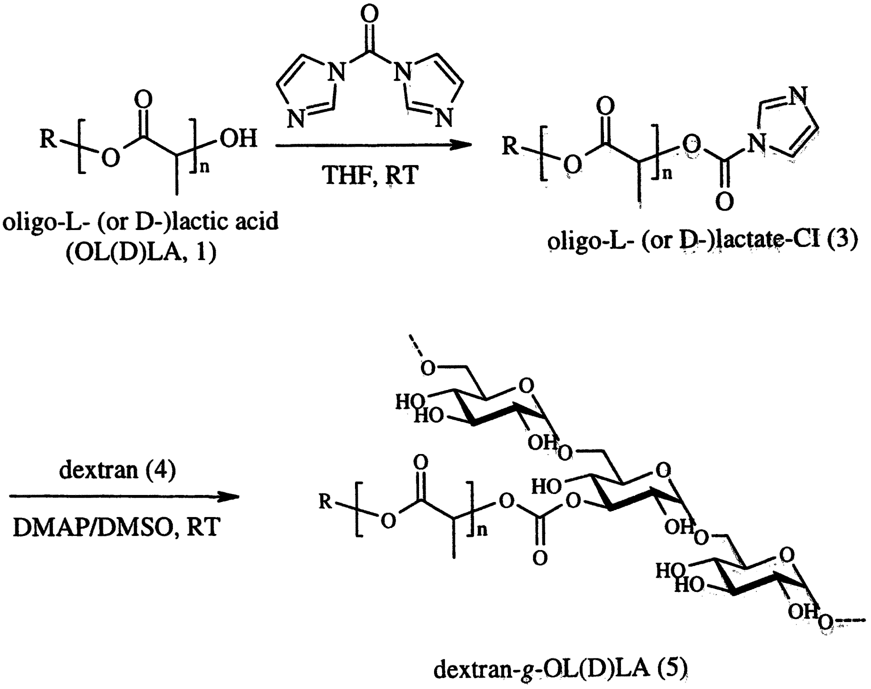
R
O
O
n
OH
N
N
O
N
N
THF, RT
oligo-L- (or D-)lactic acid
(OL(D)LA, 1)
oligo-L- (or D-)lactate-CI (3)
dextran (4)
DMAP/DMSO, RT
HO
HO
OH
dextran-g-OL(D)LA (5)

*Figure 2. Synthesis of dextran-*g*-OLA. R represents a 2-(2-methoxy-ethoxy)ethyl (MEE) group.*

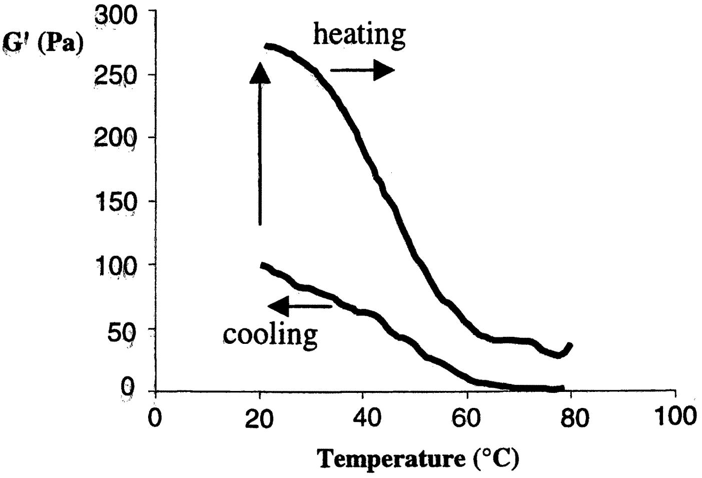

Figure 3. Storage modulus as a function of temperature of a dextran-g-OLA stereocomplex hydrogel ($DP_{average}$ = 9, DS = 3, 80 % water) upon heating and cooling. The vertical arrow reflects the increase in storage modulus in time at 20 °C to its original value. (Adapted from reference 38.)

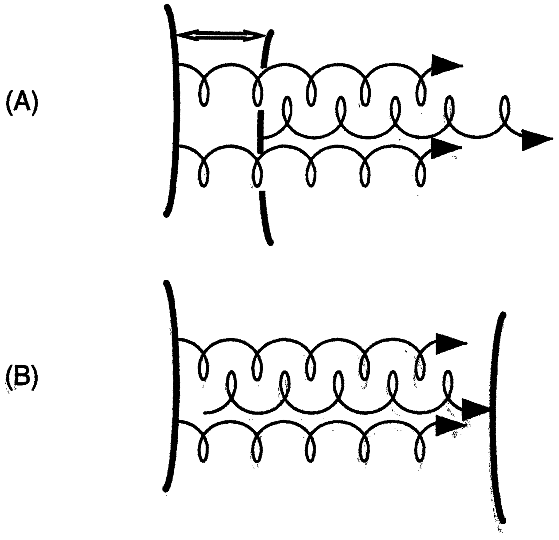

Figure 4. Schematic representation of stereocomplex formation in dextran-g-OLA hydrogels, showing the required unidirectional packing of the lactic acid chains in two cases: with both enantiomeric OLA chains connected to dextran via their OH terminus giving steric hindrance between the dextran chains (A) and one of either enantiomers coupled via the OH terminus and the other via its carboxylate terminus (B). The carboxylate termini are represented by the black arrowheads.

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hydroxyl group whereas the oligomer of opposite chirality was coupled via its carboxylic acid group.(*39*) This is ascribed to the required parallel packing of the oligomers in stereocomplexes, *i.e.* in which all the chains are oriented in the same direction (as explained in Figure 4).(*24*)

Protein-loaded hydrogels were simply prepared by dissolving the protein in the dextran-*g*-OLLA/ODLA solutions prior to mixing. It was shown that under physiological conditions the gels are fully degradable.(*40*) The degradation time depended on the pH and the composition of the hydrogel, *i.e.* the number of lactate grafts, the length and polydispersity of the grafts and the initial water content, and varied from 1 to 7 days (Figure 5). Under non-degrading conditions (pH 4) the hydrogels, having a water content of almost 90% in their swollen states, appeared to be stable for more than 1 month. As shown in Figure 6, the gels showed a release of the entrapped model proteins (IgG and lysozyme) over 6 days and the kinetics depended on the gel characteristics, such as the polydispersity of the lactate grafts and the initial water content. The release of lysozyme was by diffusion, whereas for the bigger IgG, whose hydrodynamic radius approaches the estimated mesh size of the hydrogels, also swelling/degradation played a role in the release. Importantly, the proteins were quantitatively released from the gels and with full preservation of the enzymatic activity of lysozyme, emphasizing the protein-friendly preparation method of the protein-loaded stereocomplex hydrogel.

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Conclusions

Hydrogels which are physically crosslinked by stereocomplex interactions have attracted recent attention for drug delivery purposes. Especially systems which are obtained from aqueous solutions of the two components are very attractive, since they provide a friendly environment for the encapsulation of highly sensitive bioactive substances (proteins, DNA, living cells). Moreover, it is anticipated that gel formation can take place *in situ* after injection of the low-viscous solutions. We have developed a versatile and fully degradable system obtained from water soluble dextran grafted with oligolactic acid chains. The mechanical properties, degradation profile and release of encapsulated compounds can be simply tailored by the composition of the materials. At present we are investigating means to extend the degradation time by changing the chemistry of the bonds between the grafts and the backbone. Also, the preparation of injectable microspheres is one of our goals. The biocompatibility of the system will be established, but no problems are expected in that respect since recent *in vivo* studies on chemically crosslinked dextran hydrogels already showed good biocompatibility.(*41*)

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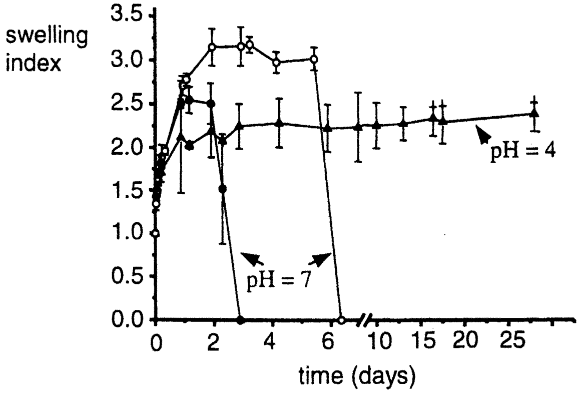

*Figure 5. Swelling behavior of dextran-*g*-OLA stereocomplex hydrogels (DS = 6, 70 % water, 37 °C): high polydispersity lactic acid grafts ($DP_{average}$ = 12, $M_w/M_n \approx 1.25$, filled symbols) and low polydispersity grafts (DP = 11 to 14, M_w/M_n = 1.01, open circles). The filled triangles represent swelling under non-degrading conditions (pH 4) (average ± s.d., n = 3 or 4). (Adapted with permission from reference 40. Copyright 2001 Elsevier Science B.V.)*

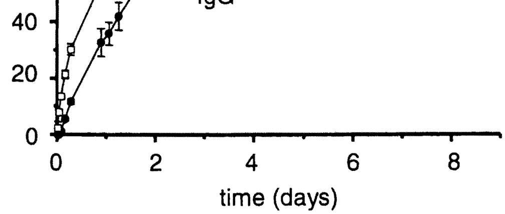

*Figure 6. Release profiles of lysozyme (open squares) and IgG (filled circles) from dextran-*g*-OLA stereocomplex hydrogel with low polydispersity grafts (DS = 6, DP = 11 to 14, 70 % water, pH 7, 37 °C) (average ± s.d., n = 4). (Adapted with permission from reference 40. Copyright 2001 Elsevier Science B.V.)*

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